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The defence of Western Europe

High-tech warfare is all the rage, and the US Army is saying that the conventional defence of Europe may again be possible. The message (which may be wrong) should not be overlooked.

Must the defence of Western Europe rest for ever on nuclear weapons, mostly made in the United States? The usual answer (yes) is a recurrent embarrassment to many of the governments concerned. In Belgium, the Netherlands and West Germany, politicians now have ample experience of the risks of defending nuclear defence. And the assumption that nuclear weapons are a necessity for Western Europe is an impediment in arms control negotiations. So it is welcome, and may be important, that in the past year there has been a revival of interest in conventional (non-nuclear) forces as a means of defending Western Europe. The interest has come from many quarters, from German military experts, from the US Army, from among technologists who are studying how the job could be done and from those more thoughtful advocates of the nuclear freeze who recognize that the West without nuclear weapons must still defend itself. The outcome could be refreshing and important.

The belief that the defence of Western Europe must be based on nuclear weapons stems from the Lisbon conference of 1952, when the embryo NATO alliance agreed that the number of troops and armaments required to counter a possible Soviet invasion were, literally, unsupportable — 96 divisions and 9,000 aircraft. To circumvent this weakness, NATO made a Faustian pact with the nuclear genie. Soviet leaders were warned that if they ever flexed their considerable muscle in the direction of Western Europe, the United States would consider its vital interests threatened and might respond with nuclear weapons — at the outset, clumsy devices carried by long-range aircraft. Since then, the basic conundrum has remained but the weapons have changed. The US weapons laboratories developed smaller nuclear weapons that could fit into guns and short-range aircraft, making tactical engagements credible, if only just; later they invented the neutron bomb, to make nuclear weapons more acceptable both to battlefield commanders and to Europeans. The most recent development was to put small but very accurately directed nuclear warheads on longer-range weapons, such as the Pershing II missile and the ground-launched cruise missile, to persuade everyone that nuclear war in Europe could be neat and precise. The US Army even called its neutron bomb the "cookie cutter".

Weapons

But no trick of technology or public relations could change the basic problem: NATO countries had mostly to rely on nuclear weapons controlled by the United States to defend themselves, and the weapons would destroy the very territory they were meant to protect.

So far, the conventional alternative has made very little headway. West European governments have consistently resisted on the grounds of cost, while the United States has pleaded in turn its commitment in Vietnam and, more recently, cost again. Only in the late 1970s did the Carter Administration exact from West European governments a commitment to increase defence spending by 3 per cent a year — handsome by past standards but still inadequate, given the build-up in conventional weapons by the Warsaw Pact countries throughout the 1960s and 1970s. So the Lisbon strategy has remained, but weakened by the Soviet strategic nuclear build-up; it can no longer be assumed that US arms will serve as Western Europe's shield.

Developments in technology and in military thinking could

now change all this quite soon. Manfred Worner, a likely candidate for the post of defence minister in any Christian Democrat government in West Germany, said in Washington last summer that NATO's previous conventional option, to increase its present 26 divisions to 36 forward divisions with 9–12 in reserve, would entail an unacceptable doubling of the West German army, the Bundeswehr. But technology, he said, may offer an alternative. An arsenal of remotely piloted vehicles, cruise missiles and shorter-range ballistic missiles, all armed with conventional munitions and terminal homing, could do much damage to the Warsaw Pact's follow-on forces that give a potential Soviet offensive punch and staying power. Such stand-off weapons could be attached to NATO's JABO-class fighter-bomber aircraft, extending the defence for hundreds of kilometres behind the main line of advance. By counter-attacking in depth, Worner argued, NATO could perhaps hold off and even turn back an invasion.

War games

US Army war games recently played at Fort Leavenworth appear to confirm this view. Using an extended counter-attack strategy with the weapons the US Army plans to have in the field by 1986, the game showed that the defence could hold an invasion through the Fulda Gap — one of the passes near the border, without resort to nuclear weapons (or to chemical weapons either). Likewise, the US Defense Advanced Research Projects Agency (DARPA) has just finished a test called "Assault Breaker" which explored what kinds of radars, missiles, munitions and terminal homing systems are available, and which combinations could defeat putative Soviet second echelon forces 100 kilometres behind the battle front. These forces, which would be expected to roll along after the first invaders in enormous timed masses, could have as many as 600 tanks, 500 armoured vehicles, 50 artillery batteries, 200 surface-to-air missiles and 300 trucks.

With the old technology, it might have taken 5,500 air sorties and 33,000 tonnes of bombs to destroy such a force. Assault Breaker showed, however, that sufficiently accurate conventional weapons with ranges from 100 to 200 kilometres could do the job as well. Tentatively, the agency estimates that to equip Central Europe with these things — many of which are already in existence but would have to be fitted onto existing planes, guns and rocket launchers — could cost a mere \$2,300 million. The calculation is that only the US forces would have to be re-equipped; allied conventional forces would be unchanged.

Such talk about the conventional defence of Europe has not been heard since the 1930s when the Maginot Line was built. An improved conventional defence, that substitutes technology and strategy for large standing armies and nuclear bombs, is an intriguing new theme. In the United States, people are talking about using dirt and concrete to fortify the passes at Fulda, Meiningen and elsewhere, forcing an invader onto the northern or southern flanks, where he would be more vulnerable. Simple things, like the construction of more short runways near the German border so that F-5s and other short-range aircraft could be brought into battle, are similarly calculated to make a big difference. If further study shows that these conventional means of defence could be effective, the result may be to liberate Western Europe from the Lisbon understanding, now thirty years



old. The consequences of that would be profound.

First, it may be possible to put the nuclear genie back into its bottle, at least where Central Europe is concerned. The governments that have struggled with opposition parties over the siting of nuclear weapons on their territory, but under US control, will jump at the prospect that conventional defence might instead suffice. And since no European government can foretell the state of public opinion after 1986, the earliest that a new-style conventional defence could be in place, even those that hitherto have been most hospitable to nuclear arms are bound prudently to look again at the new conventional alternative. Everything will depend on the degree to which they are persuaded that some version of the conventional alternative will be effective — and that the present wave of enthusiasm for it in the United States is not a blind for the withdrawal of other than strategic nuclear weapons.

Second, an improved conventional defence of Western Europe would not overnight make Central Europe free from danger. If it were possible to rely on new conventional forces to repel conventional attacks, Western Europe could still be browbeaten into submission by the threat of nuclear attack. So the promise of strategic nuclear support from the United States would still be necessary for the security of Western Europe. And those states in Europe that consider independent nuclear forces essential to their security, France and the United Kingdom, would presumably continue as at present.

The most hopeful consequence of the new interest in conventional defence is, however, in arms control. Bilateral negotiations will be resumed this week in Geneva on the Soviet and US proposals that intermediate-range nuclear weapons on either side of the German frontier should be limited, but the prospect of rapid progress is slim. The Soviet side has a tactical advantage in stringing the United States along until late next year, when the Pershing II and cruise missiles are due to be installed in Western Europe, but both sides are also genuinely perplexed to know how to strike a balance between their qualitatively very different nuclear forces. If the conventional defence of Western Europe is feasible, economically as well as technically, the bargaining position of the United States in these negotiations would be strengthened, as would the prospect that Central Europe could be made substantially safer than it is at present.

La Ronde, 1982 style

The French government may have bargained for less than it will get from a job-switch.

Will all the reforms and reorganizations of French science, which appear to have been almost continuous since May 1981, when President Mitterrand came to power, amount come to much in the end? There are two clear views in France: the cynical and the refreshing. But each observer is ambivalent.

"We've had so many, we know how to resist reforms", they say, "but there has also been a *prise de conscience*". Something does seem to have happened, but it is not so much in the rules and regulations, as in attitudes. The shift, which realigns the bulk of scientists and university staff with the economy and with industry, rather than against it as a kind of intellectual resistance movement, was under way before the socialists came to power. But Jean-Pierre Chevènement, as minister of science and now of research and industry (note the change) has confirmed the move and is attempting to institutionalize it, and with some success.

Take the National Colloquium on Science and Technology last January. "What a mess it was," said a polytechnicien the other day, "but how important!". Very little of practical importance was concluded; the committee on finance, for example, did not even consider figures, and a recommendation to preserve the French *troisième cycle* form of higher degree was ignored in Chevènement's subsequent law for research (it is billed for change). But people met at the colloquium who had never met before — in particular the universities and industry — and found they had something useful to offer each other. This is the *prise de*

conscience that led earlier this year to a large majority of university lecturers saying in response to a questionnaire that they would like more involvement with industry. This is the *prise de conscience* that is leading industry actually to pay visits to university laboratories ("I've had four visits from industry this year" said one researcher recently. "It just wouldn't have happened two years ago.") and also encouraging French scientists to seek contracts with industry.

Moreover, the recent changes at the head of the Centre National de la Recherche Scientifique (CNRS), which funds the cream of basic science in France, and of the higher education division of the Ministry of National Education, seem likely to confirm the trend. Pierre Papon, a chemist who takes over at CNRS, is somewhat quieter than his predecessor, Jean-Jacques Payan; and he has been much closer to Chevènement as a member of the minister's policy-making cabinet. Payan, at a little leaving party at CNRS headquarters a couple of weeks ago, said that "like Nelson, I know how to disobey". Papon may not be so resistant to Chevènement's plans for, say, the social sciences, where Chevènement would like to impose essentially his own view of the subject as something more akin to a tool of policy; Papon has refrained from appointing Chevènement's *protégé*, the marxist M. Godelier, to the post of director of social sciences. But clearly Papon by conviction, by inclination and by personality is likely to allow Chevènement a somewhat greater influence over CNRS than hitherto — and that will imply an inclination towards industry.

At the Ministry of National Education, the new director of higher education is Jean-Jacques Payan, the man who could disobey Chevènement. Will he protect the old interests of the universities there? Probably not. He did, after all, encourage most of the reforms of CNRS (social sciences aside); he believes in the new government, he understands the need for improving French industry, he understands the universities (he was president of the University of Grenoble) and he understands power. He is going to use that power to reform the universities along the lines of the "*prise de conscience*". The universities are highly centralized in France, and that gives Payan weapons he is not going to relinquish easily. His power is very great: he largely controls appointments and the budgets of all the universities; effectively, he is in the position of the former Minister of the Universities (when such a ministry existed), Alice Saunier-Seïté, who so appalled the universities with her dirigism, her dismantling of many *troisième cycle* degrees and what are now seen as arbitrary acts of vindictiveness.

Payan has almost the same weapons (the control of appointments has recently been made a little more democratic) and the same reforming zeal. So where does he differ from his reviled right-wing predecessor? The difference is that he is optimistic, he says, about the future of the universities whereas Alice Saunier-Seïté was ultimately pessimistic.

So the universities should, in principle, enjoy the forthcoming Payan reforms whereas they hated Saunier-Seïté's. Payan would however like to do away with the *troisième cycle*, and the subsequent *thèse d'état* (one basically a little lower, the other a little higher, than a PhD), and substitute a PhD system; and there is going to be a lot of resistance to that, unless Payan handles the situation with enormous tact — and muscle. (Many comfortable livings in French universities depend on the existence of the *troisième cycle*, which requires fewer lectures but brings in more cash than undergraduate teaching.)

However Payan is limited by one thing — the forthcoming "law for the universities", a reform of the 1968 act which established the French university system after the student rebellion of that year. This law was almost ready when Payan moved across to the Ministry of National Education from CNRS, and he may be able to make only minor changes to the existing text. But, he says, he was "pleasantly surprised" with the present version and he may have to do little to suit his taste. As for the Mitterrand government, and its use of science and technology as an economic engine: "I shall do everything in my power to make it succeed" he says.

New UK row on embryo research

Edwards in fresh ethical contretemps

Yet another public controversy has erupted in Britain about the *in vitro* fertilization of human ova and the practices associated therewith. At the weekend and in the following days, many British newspapers reported that Dr Robert Edwards, of the physiology department at the University of Cambridge, had told a weekend conference that he had been carrying out experiments on viable human embryos surplus to the requirements of *in vitro* fertilization operations.

Dr Edwards and the surgeon Mr Patrick Steptoe were the first British exponents of this technique. Most comments this week were accompanied by condemnations of what is supposed to be going on from various public figures, including Dr John Havard, secretary of the British Medical Association.

So far as can be learned, none of the popular reports so far published includes an account of what Dr Edwards actually said, first in the Galton Lecture of the Eugenics Society and then by telephone to a conference improbably held at Gatwick Airport, south of London, organized by the British Medical Journalists' Association and sponsored by the Ciba Foundation.

The occasion may nevertheless be important because of the appointment by the British government last July of a committee under Dr Mary Warnock to examine the ethical problems arising from *in vitro* fertilization. A spokesman for the Department of Health said on Monday that the newspaper reports merely confirmed that the government had foresight in setting up the committee, which is due to report two years from now. He could not immediately say whether the committee had yet met.

On the telephone earlier this week, Dr Edwards gave an account of his paper. After the Galton Lecture, the same discourse was given by telephone to the Medical Journalists' Association. He says that he explained how, in the process of *in vitro* fertilization, more than a sufficient number of fertilized ova (two or three) may be produced.

What seems not to be widely appreciated about the technique is that it is conventional to maintain these embryos in culture for between two and five days, before implanting them in the uterus of the putative mother. Dr Edwards said that he had reported to the conferences that "spare" embryos had on some occasions

been observed to provide further information on the optimum time for implantation.

Dr Edwards also reported that he told the conference that he had maintained some embryos in culture for longer than five days — nine days is the maximum so far. He said that while the primary objective of this work has been to improve the efficiency of *in vitro* fertilization, he is also interested in more distant possibilities, and told the conferences as much.

Dr Edwards said that while conventional wisdom has it that unwanted fertilized ova should be kept in a deep-freeze, but not allowed to die, until their future could be decided, there are good reasons for making use of them in studies of fertilization, differentiation and genetic abnormality.

He said, however, that there should first be "strong ethical advice" on the subject, and that those wishing to maintain human embryos for longer than a fixed period — five or nine days perhaps — should be required to have a licence to do so.

If those hurdles could be surmounted, however, Dr Edwards believes there are

substantial medical benefits ahead. While "dead against" the use of surrogate mothers to provide uterine hospitality for genetically unrelated embryos, he argues that freeze-dried congenic embryos grown at some future time to the stage at which heart or brain tissue differentiate (12-14 days) would provide adult human beings with access to compatible "spare part tissue" and thus offer an escape from immunological barriers in transplantation.

Dr Walter Hedgcock (73), a former deputy secretary of the British Medical Association, was reported by the *London Standard* on Monday as having been "horrified" by Dr Edwards's disembodied speech as received at Gatwick Airport, and to be looking for a parliamentary ban on such experiments.

Dr Edwards, not for the first time in trouble with the British popular press, considers he may have been unwise to talk to an audience without being able to look its members in the eye. The incident is nevertheless potentially important because it may prompt the British government to preempt the Warnock inquiry. ●

Reagan no science censor

Washington

President Ronald Reagan denied last Friday that his Administration sought to "close off legitimate transfer of knowledge and information" when his appointees in the Pentagon caused some hundred papers to be withdrawn in the name of national security at a symposium of the Society of Photo-Optical Instrumentation Engineers in San Diego, California, last month (see *Nature* 23 September, p.289).

At a meeting with a group of business publishers at the White House, President Reagan told me that the Soviet Union has acquired an enormous amount of US technology because of "carelessness". He defended the censorship as "just an attempt to close off those avenues where, just by reason of attendance at scientific forums and seminars, they have gone home with things that they have then turned to military advantage and the sophistication of their military build-up.

"Their technological sophistication is a threat to the whole peace-loving world . . . so that is what is back of that — not any desire on our part to close off legitimate transfer of knowledge and information."

The President continued by saying that "if, here and there, something goes too far, we will rectify that" — an apparent acknowledgement that the Administration has sometimes gone too far in censorship.

There is, however, no evidence that the withdrawal of the papers at the San Diego meeting was done with presidential knowledge, even though Secretary of Defense Caspar Weinberger had been informed in

advance of what his staff considered an impending "disaster" at the photo-optical society meeting. In view of the attendance of Soviet scientists, Mr Weinberger then asked his staff to warn those due to read papers that they might be in violation of Pentagon review procedures if they did so.

"The situation is in total confusion", says Hakime Sakai, professor of physics at the University of Massachusetts at Amherst, who withdrew his paper in response to warnings from his sponsor, the Air Force's Geophysical Laboratory at Hanscomb Field. Sakai, like others, has now submitted his paper for Pentagon review, a requirement he was unaware of until days before the meeting.

While the president stressed the importance of stopping the "careless" transfer of information to the Soviet Union, he also indicated an equal interest in preserving "legitimate" transfer of information. But neither the President nor his science adviser, Dr George A. Keyworth (who, in a statement at the time, called the photo-optical society incident "unfortunate and ill-timed"), said how these two goals will be met.

It is not yet clear which procedures cover the presentation of unclassified scientific material at international meetings or, for that matter, publication in the open literature. The office of Stephen D. Bryen in the Pentagon, which issued the warnings, cited a "new" regulation issued in April (numbered 5230.9) which requires central Pentagon clearance (instead of clearance

by contractors and sponsors locally) for any papers containing "technical data" relating to equipment having military uses. Export of such equipment and related data is regulated by the 1954 International Traffic in Arms Regulations (ITAR). But the provision has previously been applied only to such things as directions for using a machine-tool device.

The Department of Commerce, which administers ITAR, sent a telegram to scientists before the meeting warning them of possible violations of ITAR, but Professor Sakai and others were unaware of this new Pentagon regulation, or that ITAR could apply to unclassified basic research, much of it based on work already published in the open literature. Sakai believed he was in compliance with another order of the Pentagon (numbered 12356), also issued in April, which said that unclassified basic research could not be classified unless it had a "direct" relationship to national security. (Professor Sakai's paper was on infrared atmospheric emissions.)

The Department of Defense now hopes to establish a steering committee to decide what to do about future scientific meetings. Professor Sakai notes that hundreds of scientific papers are presented in the United States each week that describe work funded by the Department of Defense. "It will be a monumental problem", he says, if the Reagan Administration imposes another review requirement on all those papers, as an addition to the regular peer review that the papers now receive.

Deborah Shapley

West German energy

Two reactors in need of help

Bonn puts pressure on public utilities

Heidelberg

The West German parliamentary commission of inquiry into future atomic energy policy reported last week on the comparative safety of the 300-MW prototype fast breeder reactor under construction at Kalkar (SNR 300) and a conventional 1,300-MW high-pressure light-water reactor, Biblis B, in Hesse. Nationally, opinion about the future use of nuclear energy in West Germany and in particular about the prototype reactors is deeply divided. The commission, which included seven members of parliament and was under the chairmanship of Harald Schäfer (Social Democratic Party), reflected this split by voting 11:5 that SNR 300 does not carry greater safety risks and should be put into operation. Because there is still much to be learned about the prototype, they recommend stepwise construction and careful training of personnel. The minority view was that a maximal meltdown accident with the fast breeder reactor would have far worse effects than a similar event in a conventional reactor, and the probability of its occurrence was unknown. Political, economic and security aspects should also be taken into account.

The sodium-cooled fast breeder has been 20 years in gestation. Its first project leader

was Wolf Häfele, then at the Karlsruhe Atomic Energy Research Center, now a member of the commission. The construction firm is Interatom and the scheme is financed by the federal government, various utility companies and to a lesser extent the Belgians and Dutch.

The other prototype reactor in West Germany is the thorium high-temperature reactor (THTR 300) at Schmehausen in the Ruhr, which was originally planned to produce high-grade heat for the chemical industry and for coal distillation and which is being constructed by Brown-Boveri & Co. Although the federal government is providing 75 per cent of the costs, these have risen high enough to dampen the interest of the chemical industry, especially because the reactor seems unlikely to provide the 1,000°C needed for coal distillation.

The construction of both reactors has been dogged by bureaucracy, litigation and political prevarication. Delays cost money and it is the escalating costs that are likely to decide the final fate of the reactors.

Andreas von Bülow, the Social Democrat minister of research and technology, has been asking industry to help make up the rising deficits. He emphasized that he could not allow two projects that have got out of hand to jeopardize basic research and the support of innovations which are more important for the German economy. The total ministry research and development budget for 1982 is DM6,500

Soviet ecology

Caspian water level drops

Soviet plans to exploit the mineral resources under the Kara-Bogaz-Gol gulf have run into trouble with conservationists working on the protection of the Caspian Sea. Beneath the Kara-Bogaz-Gol lies an underground brine lake rich in sodium sulphate — an estimated 16,000 million tonnes. Long-term development plans for the Turkmenian SSR see the brines as vital feedstocks for the rapidly expanding chemical industry of the republic.

Unfortunately, the prevailing arid conditions are rapidly reducing the water level in the Caspian. The sea has an annual intake of some 300 km³ from precipitation, but loses some 355 km³ from evaporation and 30 km³ to the needs of industry and desalination plants. The Caspian thus has a net loss of 15 km³ per year. During the past 50 years its level has sunk by 2.6 m and in places the shoreline has receded by several hundred metres.

Shallow areas near the shore are primarily responsible for the high level of evaporation — in particular, the Kara-Bogaz-Gol, from which some 5 km³ of water is lost annually. Accordingly, in 1979

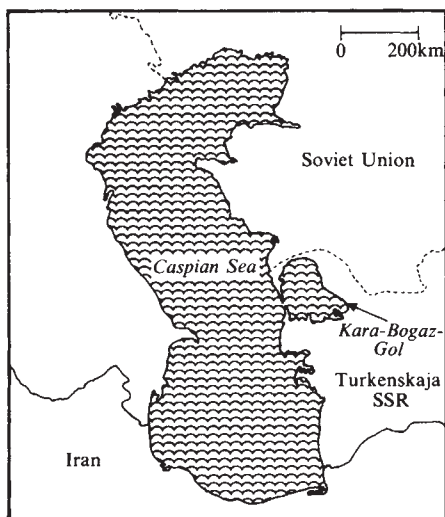
it was suggested that a dyke should be constructed across the entrance to the gulf, which would then be allowed to dry out without depleting the Caspian of water.

In spite of Moscow's enthusiasm for the scheme, others are not so keen. The leading Ashkhabad newspaper, *Turkmenskaya*

Iskra recently carried a major attack on the dam project by G. Bagirov, laboratory chief of Karabogazsulfat, the organization that exploits the brines. The dam, he said, would do immeasurable damage to the brine resources. The balance between deep and surface brines, he claims, has already been upset, so that the concentration of harmful chlorides in the deep brines is constantly rising, while that of sulphates is dropping. Moreover, if the Kara-Bogaz-Gol were allowed to dry out completely, the salts could be picked up by strong winds and ruin fertile land and fishspawning grounds for hundreds of kilometres. Meanwhile, the Caspian would gradually become more saline, as the gulf's role as a natural desalinator would be ended.

All these problems would be overcome, Bagirov claimed, if a sluice were constructed to allow limited entry of water into the gulf from the Caspian, to maintain a water balance in the gulf with at least 1 km² of saturated natural brine. A plan for such a sluice, he claimed, has already been drawn up by the All-Union Ministry of Land Reclamation and Water Conservancy, but was shelved by the Turkmenian Ministry on the grounds that more surveys were needed.

Vera Rich



million (£1,500 million), and universities and research throughout the country are on short commons.

It is relevant that in July the US General Accounting Office contested on economic grounds the priority set by the Reagan Administration on the first US fast breeder planned at Clinch River, Tennessee, at an estimated cost of \$3,500 million.

Heinz Riesenhuber, Christian Democrat spokesman on energy, has emphasized the importance of both West German reactors

for the country's industrial future. It is reported that CDU/CSU would finance the reactors by reallocating research funds. The commission's report goes to the Bundestag this week, but voting may await resolution of the current Bonn crisis. The reactors' future would be safest with a CDU/CSU coalition (their main candidate for minister of research and technology is Albert Probst — chairman of the intra-parliamentary research committee).

Sarah Tooze

UK nuclear power

Decision time

The management style of the United Kingdom Atomic Energy Authority may be subtly changed from 1 October, with the accession of Sir Peter Hirsch, the Oxford metallurgist, as the authority's part-time chairman. Since 1967, the successive chairmen of the authority (Sir John Hill and Sir Walter Marshall) have been full-timers from the nuclear industry.

Most immediately, the change will probably mean more responsibility for Mr A. M. Allen, deputy chairman of the authority for the past year and previously responsible for finance and administration. Mr Allen, who describes himself as a "lapsed historian", is now the authority's chief executive. Whether and to what extent Sir Peter Hirsch will think it politic to follow his predecessors in outspoken advocacy of a strong British nuclear programme remains to be seen.

In the public inquiry on the proposed pressurized water reactor for Sizewell (Suffolk), which is due to get under way early next year, the running is likely to be made by the Central Electricity Generating Board, the purchaser of the plant. The authority's chief concern is the programme of fast reactor development centred on the Prototype Fast Reactor at Dounreay, now costing close on half the authority's net budget (£201 million in the year ending last March).

The Department of Energy has made encouraging noises in the House of Commons about the continuation of this programme, which among other things is an important source of employment in the north of Scotland. The authority's annual report, published this week, says that problems with leaks in the steam evaporators (through which molten sodium from the reactor core circulates) continued during the past year, reducing the power output of the reactor and thus the amount of spent fuel available for reprocessing through the experimental plant at Dounreay.

The next milestone for the British fast reactor programme will be the government's decision on the proposal to build a commercial demonstration reactor, the capital cost of which is now estimated at 20 per cent more than the Advanced Gas Cooled Reactor being built at Heysham. The government has been brooding about this decision for at least the past two years.

The possibility of partnership with the Commissariat à l'Energie Atomique on the Superphénix programme seems to have receded as the French programme itself has faltered. Nobody seems to know how to devise a framework for Anglo-American collaboration that would survive the confusion about the Clinch River fast breeder reactor.

In the end, much will depend on how much the Central Electricity Generating

CERN

Electrons collide environmentally

The European Centre for Nuclear Research (CERN, near Geneva) is still battling with the politics of its next big project the large electron-positron collider, LEP. Although LEP was officially approved at the international CERN Council last December, local French opposition has again reared its head. But CERN is in such a hurry to get LEP built that it seems prepared to ignore opposition, and even, effectively, to 'gag' members of its own staff.

The problem is "environmental" approval. LEP must meet the regulations of France and Switzerland, through both of which it passes. Swiss law is already satisfied. But the French environmental hearings have only just started, and strenuous objections are already coming from the little village of Echevenex, tucked under the Jura Mountains six miles from the present CERN site.

Some 30 CERN staff live in the village, which is a well-known thorn in the CERN flesh. Last year the mayor, M. Honorat, managed to halt construction of a pilot tunnel under the Jura, and was finally overruled by the highest French legal body, the Conseil d'Etat. Since then both LEP and M. Honorat have changed their positions — LEP to avoid construction problems under the Jura, eliminating the need for the tunnel, and M. Honorat for reasons of his own. LEP appeared to be safe.

However, the new path of LEP, although out of the Jura, comes much closer to Echevenex — and brings with it one of the four primary experimental halls, which will appear on the surface like a 400-metre-long factory, only 250 metres from the edge of the village, which looks down on the site across a valley. "It's a beautiful spot" said one resident last week. "The hall would go where the children like to play, and where we pick nuts". The site would be an eyesore in what is essentially a rural retreat, say the villagers — and it would bring noise.

The objections may be dismissed as romantic — unless one lives there — but CERN director-general Professor Herwig Schopper wrote recently that "We should aim not merely at LEP being tolerated by the local inhabitants, but at making them proud supporters of CERN as a positive

asset in their neighbourhood". Echevenex villagers see little sign of those ideals. At a meeting with CERN officials in the village on 2 July this year, the CERN reply to a question about noise was "you'll get used to it".

This apparently unhelpful attitude incensed even CERN employees in the village, and one of them, say the villagers, began to raise technical questions. For his pains, however, the employee was carpeted, and his attention drawn to a clause in the CERN employment contract which reads "Members of the personnel shall refrain from an act or activity which is incompatible with their functions or which would be materially or morally prejudicial to the organization". Clearly frightened for his future, he withdrew from the discussion. Since then, CERN has published a notice which indicates that employees are free to act in their interests, within the national law of their place of residence — but that they must also take note of their conditions of employment; thus, if they criticize CERN they are in danger.

What happens next depends on how CERN chooses to react, and on the process of French law. According to CERN foreign relations director, M. Lévy-Mandl, CERN is likely to make "only small adjustments" to its plans. It would be technically possible to move buildings, he said, but this would slow the rate at which experiments could be moved in and out, compromise safety and increase costs. Representations to this effect would be made to the Commission of Inquiry, a body of six local personalities now taking local views on LEP according to French law. This commission will present its conclusions to the French government, which must then decide whether or not to give LEP the status of being "in the public interest"; construction could then go ahead.

It seems inconceivable, now, that approval will not be given — so the question facing the government, with its professed concern for regionalism, is simply how far to take into account the objections of the little village of Echevenex. Will the Echevenex halls be just where CERN says they should be? Or where the villagers would like them?

Robert Walgate

Board offers to contribute to the cost. Meanwhile, the authority plans to use the prototype reactor, commissioned in 1974, for further studies of the fast reactor fuel cycle.

Against the general trend, the authority increased its skilled work-force by a hundred to 2,925 during the past year, but expects now to be stationary. There seems to be some concern that the Harwell establishment may not be able to sustain its

present level of contract work for industry, worth more than £25 million a year.

The authority's annual accounts, traditionally among the most laconic of public corporations', are further made obscure this year by the large increase (of

£40 million) in the net cost of operations in the year ending last March, partly attributable (to the tune of £12.5 million) to the cost of writing off the capital cost of miscellaneous fissile material and equipment.

Internees in Poland

Psychiatric abuse claim

Four political internees in Poland have appealed to the World Psychiatric Association, claiming to be victims of psychiatric repression. The four had been temporarily transferred from internment camp to the municipal hospital in Lowicz for treatment in the departments of surgery, internal medicine and laryngology respectively. On 18 August, however, their treatment was discontinued, and they were transferred to the psychiatric wing of a hospital at Zgierz (near Lodz) and placed in the ward for alcoholics. Their letter appeals to the World Psychiatric Association for "speedy intervention".

As the claimants themselves point out, this appears to be the first case of psychiatric reprisals against internees. One Solidarity activist, the logician Jan Waszkiewicz, who was arrested on charges of organizing a strike in the Lenin shipyard in Gdansk and of continuing union activities after the proclamation of martial law, was sent by the court to a mental hospital after the major charge of organizing the strike had been dropped. Friends of Waszkiewicz have said, however, that he seemed to be in a genuinely disturbed state, and that there was nothing sinister in his being transferred to the hospital, where he seems to be undergoing a normal rest cure. He is not receiving any medication or other treatment and is allowed to leave the hospital once a week.

The use of psychiatry as a means of political reprisal has always been abhorrent to Polish psychiatrists. In 1975 there was considerable concern that proposed new mental health legislation would make easier the incarceration of political dissidents in mental hospitals. But after a public outcry, the proposed bill was dropped and Polish psychiatrists — unlike their colleagues in the Soviet Union — avoided the possibility of having to diagnose political dissent or religious enthusiasm as a symptom of schizophrenia.

During its five years' existence (1976–1981), the Workers' Defence Committee KOR, which documented abuses of human and civil rights in Poland, recorded only one attempt at psychiatric confinement — the case of a young man petitioning for the broadcasting of Sunday Mass — and this fell through because psychiatrists were unwilling to certify him.

Under martial law, however, the security services have taken renewed interest in the possibility of psychiatric internment. After

the demonstrations over the May Day weekend, according to underground Solidarity sources, the commandant of one of the departments of the mid-town district of Warsaw, Magistrate Czepinski, applied to Dr Zielinski, director of the Nowowiejski hospital, for facilities for the hospitalization by the civil police of "mentally deviant persons who exhibit a tendency to disrupt public order when various festivities are organized".

Dr Zielinski refused to comply with this request, and reminded Czepinski that the law on psychiatric admissions permits the hospitalization of a mentally sick patient only at his own request, the request of a close relative, or, under carefully defined conditions, by a general practitioner.

The Zgierz incident, however, indicates that not all Polish doctors have the courage and quick-wittedness to follow his example.

Vera Rich

Cryo-transmission microscopy

Fading hopes

Bonn

The three German laboratories developing an electron microscope to operate at the temperature of liquid helium have jointly agreed that at least one of the supposed advantages of their machine is not nearly as great as originally thought. That conclusion, however, is based on observations with only one crystalline sample, and opinions differ as to how serious a setback it represents.

At 4 K, the cryo-transmission electron microscope (cryo-TEM) has the theoretical advantages over conventional machines that molecular movement should be damped and damage to the sample from the beam of electrons minimized. Initially, the reduction in beam damage was estimated to be only modest, so that there was considerable excitement a few years ago when Dr I. Dietrich's laboratory at Siemens AG in Munich — where the superconducting lens for the cryo-TEM was developed — claimed that beam damage at 4 K could be reduced by as much as several hundredfold.

Now the Siemens group has agreed with Dr Jacques Dubochet's group at the European Molecular Biology Laboratory (EMBL) in Heidelberg that the protection gained by working at 4 K compared with room temperature is not more than tenfold for standard samples of L-valine.

A few weeks ago, the two groups

Electronics giant

Brussels

While Mrs Margaret Thatcher was in Japan expressing the United Kingdom's concern at Japan's excessive imbalance between imports and exports and attempting to encourage cooperation between Japanese and British high technology companies, another strategy for coping with Japan's exports of electronic goods was under discussion in Brussels. Max Grundig, the founder of the German electronics group that carries his name, recently put before EEC Industry Commissioner Etienne Davignon and Karl-heinz Narjes, the European Commissioner from West Germany responsible for innovation and the internal market his own solution for improving the competitiveness of the European electronic components industry.

Grundig proposed that the European Commission should support a European company for the development and production of electronic components, bringing together the resources of Grundig-Telefunken, Blaupunkt-Bosch and ITT-Schaub-Lorenz. Other European producers would be welcome to join in, with the exception of The Netherlands' Philips and the French group Thompson — the company might be too large and unwieldy if such industrial giants were included.

Grundig is anxious to gain EEC support for his idea in order to side-step both German and EEC anti-trust and monopoly laws. Grundig argues, however, that something must be done soon to meet the threat of Japan's rapidly growing dominance; otherwise he fears there will be grave repercussions for both the consumer electronics industry and for Europe's defence technology companies. He would like his project to be launched as soon as next spring.

The idea of an aggressive new European electronics company met with a warm reception in EEC circles, although there will have to be many further discussions before anything is decided. A meeting has, however, already been arranged of representatives of leading European manufacturers at which Davignon will take the chair.

Jasper Becker

together with that of Dr E. Zeitler of the Fritz Haber Institute in Berlin (the only other recipient of a Siemens superconducting lens) agreed, within limits, on a revised estimate of L-valine beam damage at 4 K. A brief statement of their measure of agreement has been sent to the *Journal of Molecular Biology* which in 1980 published an estimate (by Dr E. Knapek of Siemens and Dubochet) of a cryo-protection factor of about 70 for L-valine.

The note implies that beam damage can vary greatly with specimen preparation, and especially with the characteristics of the material that supports the sample. Dr Knapek also believes that orientation of the sample and support relative to the electron beam is important, and may account for the discrepancy between the original and revised cryoprotection factors. A more

rigorous test of cryoprotection will come, he feels, from a planned comparison of four further compounds whose orientation is known.

One of these specimens will be the "purple membrane" of a peculiar bacterium. The results will be anxiously awaited at EMBL, which has devoted much time and effort to the development of a cryo-TEM. Even if the cryoprotection factor turns out to be minimal, the decrease in molecular movement at 4 K should still improve resolution to a worthwhile degree.

But nobody now confidently predicts that the cryo-TEM will be much better than existing electron microscopes for the study of biological samples. And it is hard to know if the scanning version of the cryo-electron microscope will be any better.

Peter Newmark

Genetic manipulation

What future for regulation?

UK committee's role disputed

The future of the UK Genetic Manipulation Advisory Group (GMAG) remains uncertain; its third report, published on 24 September (Cmd 8665, HMSO, £7.75), concludes that health risks involved in recombinant DNA technology are and are likely to remain negligible. GMAG's terms of reference remain the same as when it was set up in 1976 except that it must now submit annually, and not when the fancy seems to take it. Despite a formal pat on the back in the foreword from Sir Keith Joseph, Secretary of State for Education and Science, and a note in the report that long-term monitoring is still necessary, GMAG seems on the point of working itself out of a job.

The Association of Scientific, Technical and Managerial Staffs (ASTMS), which has three members on GMAG, is clearly alarmed not only that GMAG will not survive but that its terms of reference are no longer appropriate. A delegation of six members of parliament sponsored by ASTMS and the three ASTMS representatives on GMAG — Donna Haber, Professor Derek Ellwood and Professor Bob Williamson — met Mr William Shelton, the minister responsible for GMAG, last week to put the union's case.

The delegation pointed out that GMAG had played an important role in ensuring the safe development of genetic manipulation and in reassuring public opinion. It argued that despite the successful identification of potential health and safety risks, there remained some areas where knowledge is limited and which would need to be carefully scrutinized case by case.

The delegation also pressed for an extension of GMAG's role to cover the

ethics of human genetic engineering. ASTMS says that gene replacement therapy will affect the population as a whole and should not be decided by hospital ethical committees alone. ASTMS also wants GMAG's function to include problems of environmental contamination which could result from the large-scale use of genetically manipulated organisms. The union feels that GMAG has kept up a dialogue with industry and is well placed to advise on the safety of industrial biotechnology.

The minister appears to have told the delegation that the government is fully aware of the issues in genetic engineering, especially because biotechnology would make an important impact on industry in the next five years. But he insisted that GMAG is not an appropriate body to advise on ethical issues.

The GMAG report notes that the group's advice on the large-scale use of genetically manipulated organisms would be restricted to the biological properties of the organisms in use. The Health and Safety Executive (HSE) will be concerned with the physical aspects of safety. In this spirit, GMAG seems to have delegated responsibility for site visits to laboratories in the past year entirely to HSE.

On the future of GMAG, it is planned that a full meeting of the group will discuss various options in November. GMAG members are themselves divided. Some see the group's importance as residing in its capacity as public watchdog and reassurer. Many feel that GMAG was set up to advise on the safety of certain experiments and that its remit should end there. Others point to the existence of local safety committees and their dwindling workload as evidence that the group has become redundant. The government may well share this view and reorganize GMAG into an expert committee.

Jane Wynn

Greens gain in Hesse

Heidelberg

The Hesse election on 26 September provided the latest in a string of surprising events in West German politics. Upsetting all predictions, the Christian Democrats (CDU) and Social Democrats (SPD) emerged neck and neck with 52 and 49 seats respectively in the regional parliament. The Green environmentalist party (see *Nature* 17 June, p.528) will be represented in the Hesse parliament for the first time with a crucial minority of nine seats. The Free Democrats (FDP), whose decision to withdraw from national coalition with the SPD may cause the collapse of Schmidt's government, have lost their seats. SPD, under Ministerpresident Holger Börner, continue as a minority administration, but the likelihood of Green/SPD administrative and legislative cooperation are slimmer in Hesse than in Hamburg.

The Greens' success rests mainly on local environmental issues, of which Hesse has more than its fair share: the new runway addition to what is already Europe's largest airport at Frankfurt, plans for a reprocessing plant for nuclear waste, plans for a third 1,350-MW nuclear power plant at Biblis where a 2,600-MW capacity plant already exists, US military poison gas depots, pollution of all kinds and so on.

The Hesse result has much to do with contrast between Helmut Schmidt's cool statesmanship and the less dignified politicking and fiscal sabre-rattling among FDP, CDP and CSU since the SPD/FDP coalition broke up nine days earlier; nevertheless, the Greens, now represented in six regional parliaments, are indisputably the third national party. The establishment is beginning to take them seriously — after the election result was announced, Willy Brandt talked about a left-wing alliance between the Greens, the trade unions and the SPD left. The Greens themselves, however, are unwilling to compromise on what they consider to be the key issues, such as nuclear power, armaments and industrial development, and are wary of being absorbed and corrupted by the corridors of power and by the blandishments of SPD in particular.

There are clear national implications: CDU/CSU and SPD have no electoral mandate for even a temporary administration, national elections must take place soon and there will be Green seats in the tense Deutschen Bundestag. The date for the federal election may be determined on 1 October and an outcome like that in Hesse and Hamburg is a real possibility, with the balance of power lying with the environmentalists.

Sarah Toozie

US international cooperation

Science board's other voice

Washington

The National Science Board, the governing body of the National Science Foundation (NSF), has just issued a stirring but gently worded plea for continued scientific contacts between the United States and other countries, including the communist bloc. The board also called on NSF director, Mr John B. Slaughter, to "play a significant role, with the Department of State and the Executive Office of the President, in development and implementation of international science policy".

The board thus authorized Slaughter (who plans to leave his post at the end of the year) to become involved in decisions on international science that are now usually made by the State Department and the White House without consultation with NSF. Although NSF manages several major international science programmes, such as the Deep Sea Drilling Project and the US Antarctic Program, historically NSF has had little influence over diplomatic and policy decisions, such as that made by the President after the imposition of martial law in Poland last December to terminate bilateral US-Soviet scientific agreements as they came up for renewal. The decision effectively ended most US-Soviet scientific contact.

The National Science Board, which is second only to the White House Science Advisory Committee in authority and prestige, has only rarely played a role in these decisions. "The board's options are quite limited" explained one member involved in drafting the recent statement. On the one hand, he explained, it advises NSF and is part of the executive branch of government. In this capacity it recognizes that there are situations in which the government might legitimately want to classify scientific data. On the other hand, the board is made up of, and reflects the thinking of, US university scientists, who believe that science fares best when it has plenty of international freedom.

The statement reflected this difficulty, noting that "restrictions which diminish that openness are likely to have serious costs to science and, ultimately, to national security. Such costs should be carefully considered . . . before implementing any actions that would compromise the traditionally open environment that has served us so well in the past."

The board's plea for internationalism comes just a month after the Department of Defense blocked the reading of about 100 papers at a San Diego conference (see this issue, p.383, and *Nature* 23 September, p.289) — the most recent example of the Reagan Administration's campaign against the flow of scientific information to communist countries.

● The number of US participants in international scientific congresses has been declining, even as US science has become more dependent on work done abroad, as the table and figure show. Moreover, the latest data, for 1980, shown in the table, exclude information on the growing list of international congresses at which no US scientists participated.

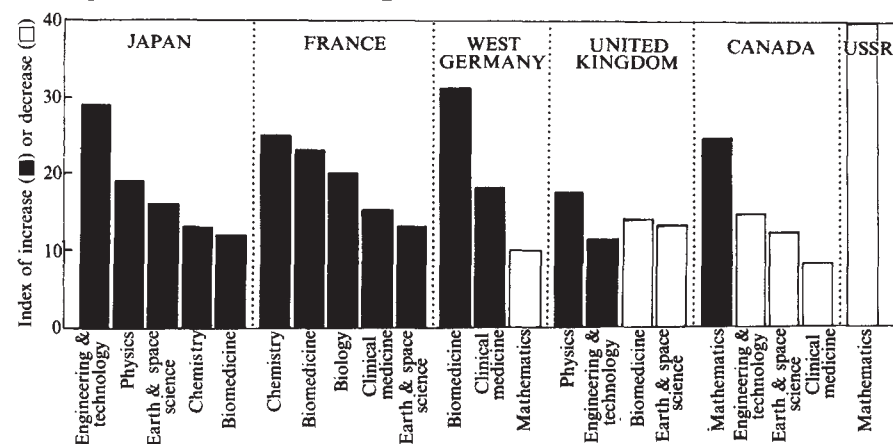
US participation in international scientific congresses			
Year	No. of congresses	Total participants	No. of US participants
1960-62	23	33,082	9,033 (27%)
1963-65	28	37,964	10,012 (26%)
1966-68	42	59,748	12,297 (21%)
1969-71	38	55,711	12,956 (23%)
1972-74	73	73,819	18,630 (25%)
1975-77	52	59,658	12,767 (22%)
1978-80	37	55,358	7,975 (14%)

Source: *Science Indicators-1980* and NSF unpublished data for 1980.

At the same time, US scientists are depending more and more on foreign scientific work in selected fields, as indicated by citation data shown in the figure. The board's action, therefore, comes at a time when US scientists are relying more than ever on their foreign colleagues — but are seeing less and less of them.

Deborah Shapley

Changes in US use of foreign scientific literature, 1973-79



Planetary science

Shopping list

Planetary scientists in Europe have been getting together. Under the umbrella of the space science committee of the European Science Foundation (ESF), they have recently published a report which recommends that the European Space Agency and national organizations take action to strengthen their subject.

Europe's planetologists seem somewhat surprised at their own level of expertise, given that they have had fewer opportunities than their Soviet or US colleagues to participate in deep space missions. Their report is an attempt to ensure that they at least maintain their international status. But the Executive Council of ESF, which has yet to decide how to advocate the report's recommendations to ESA and national governments, may have a tough task. Funding agencies, now everywhere short of cash, will be asking what level of priority to assign to planetary science compared with other branches of space science and astronomy.

The planetologists, however, seem aware that straightforward requests for more missions will fall on stony ground. Hence, they recommend that ESA, for example conduct more preliminary studies of missions, thus ensuring that final decisions on which to argue are well-founded. Money might also be saved, says the report, if the spacecraft design for Giotto, the European mission to Halley's comet, is adopted wherever possible in deep space missions.

Planetary science could also be strengthened from the ground, says the report. There should be better access to optical and radio telescopes and long-baseline interferometry. New instruments should be developed through greater collaboration between research laboratories and manufacturing industry and cooperation between space scientists should be encouraged to "take full advantage of facilities such as the space telescope".

ESF itself may have the power to act on two of the report's recommendations: that studentships be set up for young planetologists in centres of excellence and that a system of travel grants be available for European planetary scientists to visit each others' laboratories.

But the most significant outcome of the European planetary scientists' recent get-together may be apparent after a meeting with their US counterparts next October. Under discussion will be joint missions probably too costly for one agency to carry out alone. Ideas, which are still very preliminary, include missions to Venus and Mars and a mission to Saturn and the outer planets. The plan is to make a choice within the next year in time for launch in 1991.

Judy Redfearn

Video education

US engineers forge ahead

Washington

The poor track record of video-based education in the United States is showing signs of improvement. Mid-career engineers employed in US companies are now benefiting from the country's first widely successful experiment in video education.

Orders have been mushrooming for engineering and related courses to be taught by video this year and compiled and catalogued by a little-known group based in Atlanta, Georgia. Although the Association for Media-Based Continuing Education (AMCEE) is only two months into its current fiscal year, it has as many orders now as it had halfway through the previous year. John T. Fitch, the group's executive director, estimates that some 30,000 to 40,000 people, most of them engineers although they include businessmen and secretaries, are taking AMCEE-offered courses.

AMCEE serves as a nonprofit-making clearinghouse for courses produced at universities around the country. Its member universities now number 22 and include many state institutions and private schools.

To qualify, a school must have a campus-based media-based continuing education programme. The programme films the professor lecturing to students — or in a campus studio — packages the cassettes and related text material, and distributes them, weekly or biweekly, to companies, usually in the immediate area. This year's AMCEE catalogue contains 448 courses in 18 disciplines given at 21 universities. Course contents range from "Grammar for Secretaries" to "Advanced Heat Flow Analysis".

The latter course, taught by Professor W. Edwards Deming of Massachusetts Institute of Technology and consisting of 14 videocassettes and a textbook, has been a best-seller. In it, Deming teaches, according to the AMCEE catalogue, why "productivity increases as quality improves" in industrial production, and explains the "14 steps that must be adapted to achieve this result". There have been at least 4,000 orders for this course.

Some of the participating schools have raised continuing education for engineers by video to an advanced art. Colorado State University, for example, offers 76 video courses, most taken by students who are employed at companies in the Denver-Boulder area. Couriers drive the taped cassettes around to the locations where the students are taking the courses — usually at their employers' offices — and pick up homework assignments, questions, and the like.

AMCEE's success — which is in marked contrast to many other attempts at systematized video learning for credit — is based, says Fitch, on a tradition in engineering that calls for working engineers to go back to school, to catch up on basic knowledge that has advanced since they graduated, or to retrain for new specialties. Traditionally, companies give their employees leave to go back to school for this retraining, but this is expensive in terms of lost pay, commuting costs, and tuition. The video system means that the engineer can take the course at his office, while remaining employed.

So far, AMCEE does not offer any courses for undergraduate engineers. However, it does have big plans. Under Dr Lionel Baldwin, dean of engineering at Colorado State University, AMCEE is drawing up plans for schools to offer courses by satellite in real time, so that the engineer who has moved with his job to Massachusetts can take a course offered at Stanford and talk to his teacher by satellite. The proposed "National Technical University" could offer an accredited degree — all by video.

If the plan materializes, it will be a big step for AMCEE, which was embryonic after its founding in 1976 until grants from the National Science Foundation and the Alfred P. Sloan Foundation helped it get started gathering and vetting course materials.

Although a study by the Corporation for Public Broadcasting published last year estimated that half a million people around

the United States take some kind of course using television, specific programmes of video education have not usually fared well. At the end of this month, for example, the University of Mid-America (UMA) goes out of business; it was an



eight-year attempt, funded by the federal government and supported by 11 state universities, to make and distribute video courses around the mid-west. At present, says Milan Wall of UMA, around 8,000 students take courses that use UMA-developed materials, which range from a history of the Great Plains to subjects in the humanities. But UMA's market — housewives seeking more learning and adults who dropped out of college but who now want to complete their degrees — has not materialized. Even direct distribution of British Open University programmes has not been extensive, Wall says, "not because they're not of high quality but because they're designed for a totally different educational system."

Deborah Shapley

China's intellectuals back in fashion

China's new policy of eliminating traces of "leftism" which led to the recent abolition of the post of Party Chairman, will mean an upgrading of both status and living standards for intellectuals. A number of party and government documents, mostly at provincial level, have recently called for educational campaigns, intended to "surmount the traditional stance of looking down on intellectuals".

Proposed measures include:

- The complete rehabilitation of all intellectuals unjustly or falsely persecuted during the cultural revolution, including the restoration of or compensation for lost salaries, and the "education" of those involved in the " mishandling " of such cases.

- Opportunities for employment for intellectuals in accordance with their talents and qualifications. Intellectuals with "ability and integrity" who are in the "prime of life" should be selected for key posts, and their opinions on economic policy listened to "conscientiously". Party cadres are instructed to respect non-party intellectuals, while factories and other organizations assigned new graduates "must not haggle" about giving them

appropriate employment.

- Intellectuals should be able to spend five sixths of their working time on professional activity; facilities (including study leave) must be provided for them to keep up their knowledge.

- The system of examinations and promotions for professional and technical grades and titles should be improved. Allowances for education and research in provincial budgets should be gradually increased and the provision of equipment, instruments, books and auxiliary staff ensured. Party schools should offer scientific and technical courses. Administrators in key posts whose own academic or technical background is not up to standard should be sent on refresher courses.

Some officials, it is noted, have not recognized the importance of intellectuals in the community, considering that once the intellectuals in their particular area have been rehabilitated, all is well. Parents, however, have been quick to grasp the new trend and a recent article in the party journal *Red Flag* criticizes parents who have already started cramming their three- and four-year old children in preparation for university entrance.

Vera Rich

CORRESPONDENCE

Tanks are unsafe

SIR — The discussion about the effectiveness of "enhanced radiation weapons" (ERWs) against vehicles such as tanks brought up the question of whether advanced radiation shields in the armour of tanks could substantially improve the protection factor^{1,2} against radiation from ERWs.

Tables 1–3 give the results of a series of shielding calculations. Based on the methods used in ref. 3 (for example, the use of code ANISN), we assumed an explosion height of 500 m.

Three different shields have been proposed¹ incorporating a strong neutron poison into the sandwich structure of the armour of modern tanks. Each shield is based on a layer of a strong neutron absorber (B_{nat} and 6Li), and they differ only by the amount of neutron absorber present in the shield (0.1 cm B, 0.5 cm B and 1 cm 6Li_2O).

Total shielding is defined by:

$$C_{tot} = \frac{(D_n + D_\gamma)_F}{(D_n + D_\gamma)_B}$$

where D_n and D_γ are the neutron and gamma radiation dose rates in rads and subscript F refers to the layer in front of the shield and B, that behind the corresponding layer. Neutron and gamma shielding are defined by:

$$C_n = \frac{(D_n)_F}{(D_n)_B} \quad \text{and} \quad C_\gamma = \frac{(D_\gamma)_F}{(D_\gamma)_B}$$

The tables clearly show that the neutron

radiation determines the total radiation dose in all cases.

To determine what effect various cross section libraries have on the shielding factors, three different cross section libraries were used (ENDFB IV, VITAMIN C and LASL), and the differences were insignificant (Table 1).

The total protection factors of the classical armour of tanks (about 10 cm steel wall) lay in the range 2–2.5, and the relatively complicated and heavy shields considered here exhibit a total protection factor between 6 and 7. Even for a 1-cm thick 6Li_2 layer, the shielding factor does not increase substantially. The physical reason is, of course, that the neutron spectrum is too broad to make the neutron poison an effective absorber in all energy levels.

Thus still more moderator material would be necessary to guarantee a sufficiently high protection factor. The protection factor should be of the order of magnitude of 1,000 to reduce a dose of 20,000–30,000 rads to a few tens of rads. This dose could be survived without the biological and psychological effects of a radiation illness.

Water is a cheap and practical shield material for protection against radiation from ERWs. Assuming an explosion height of 500–1,000 m, the half-value thickness of water is about 15 cm and a very thick water shield would be needed for the required protection factor.

In conclusion, it seems impossible to design a practical shield for manoeuvrable tanks with a sufficiently high protection factor — such a

shield would be too heavy. This confirms our previous findings³ that protection against radiation from an enhanced neutron weapon is much easier (and cheaper) for the civilian population than for tank crews.

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2. Gsponer, A. *La Bombe à Neutrons est-elle une arme antichar vraiment efficace?* (Geneva International Peace Research Institute, GIPRI-82-05, 28 February 1982).
3. Köhler, P., Seifritz, W. & Stepanek, J. Protection against Enhanced Radiation Weapons in *Atomkernenergie* (in the press).

Falling stock

SIR *Nature* has decided to apply one of its news pages to a biotechnology stock report (*Nature* 12 August, p.599). As a financial analyst who specializes in biotechnology, I must question the need for printing information such as this in a professional journal of science. It would be rather like a drug stock index in *The Lancet*. In addition, I find the selection of stocks arbitrary. Collagen Corporation is a superior company and a good investment, but what does conversion of two cowhides per month into pure, injectible collagen have to do with "biotechnology?" And in the chauvinism department, why exclude such Japanese Corporations as Ajinomoto, Kyowa Hakko and Green Cross? More seriously, the stock index is so heavily weighted by Novo Industri A/S and AB Fortia, two Scandinavian companies, that perhaps you could perhaps call it the "*Nature* ScandIndex".

SCOTT R. KING

Brooklyn, New York, USA

Polish defection

SIR — The article "Norway's Arctic diplomatic fix" (*Nature* 9 September, p.97) grossly misrepresents the development in connection with the defection of two members of the Polish geophysical station in Svalbard in August 1982.

According to a report to the local newspaper in Svalbard, the "*Sysselmannen*" — governor of Svalbard — sent a helicopter to the station on 3 August to collect a station member who had asked for political asylum in Norway. On 10 August another member defected. There was no "race" between Norwegian and Soviet helicopters, and no intervention by Soviet helicopter crews or by the Soviet authorities in Barentsburg in order to prevent the defections.

Since the article also refers to the duties of our institute in relation to foreign expeditions, we would like to point out that it provides information on the scientific activity in Norwegian Polar areas and also on governmental laws and regulations, but has no obligation to give free access to its scientific material. In due time, the data are published in our own scientific series, a monograph series, *Skifter*, and a bulletin, *Polar Research*, or in international periodicals.

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Table 1 Neutron-, gamma- and total shielding factors of a 0.1-cm boron-containing shield

Material sequence of shield	C_n			C_γ			C_{tot}			D_n/D_γ		
	A	B	C	A	B	C	A	B	C	A	B	C
500 m air	1	1	1	1	1	1	1	1	1	7.72	7.38	6.23
5 cm steel	1.94	1.93	1.94	1.27	1.35	1.31	1.83	1.84	1.81	6.03	5.15	4.21
10 cm water	6.05	6.16	6.17	1.87	1.96	1.99	4.82	4.91	4.78	2.38	2.35	2.00
0.1 cm boron	6.15	6.35	6.37	1.91	2.01	2.03	4.91	5.05	4.92	2.4	2.33	1.99
1 cm lead	6.53	6.75	6.75	3.83	4.11	4.01	6.04	6.27	6.17	4.52	4.5	3.7

Values assume an ERW explosion at a height of 500 m. Three different cross section libraries were used in the calculations (A, ENDFB IV; B, VITAMIN C; C, LASL). The last column gives the neutron- to gamma dose ratios at the various positions within the shield.

Table 2 Neutron-, gamma- and total shielding factors of a 0.5-cm boron-containing shield

Material sequence of shield	C_n	C_γ	C_{tot}	D_n/D_γ
500 m air	1	1	1	7.39
5 cm steel	1.94	1.35	1.84	5.16
10 cm water	6.20	1.96	4.93	2.33
0.5 cm boron	6.53	2.17	5.27	2.46
1 cm lead	7.03	4.30	6.53	4.52

Values assume an ERW explosion height of 500 m. VITAMIN C nuclear cross sections were used. The last column gives the neutron- to gamma dose ratios at the various positions within the shield.

Table 3 Neutron-, gamma- and total shielding factors of 6Li -containing shield

Material sequence of shield	C_n	C_γ	C_{tot}	D_n/D_γ
500 m air	1	1	1	7.39
5 cm steel	1.94	1.37	1.85	5.24
10 cm water	6.28	2.21	5.15	2.6
1 cm 6Li_2O	6.77	2.48	5.61	2.71
1 cm lead	7.29	4.47	6.78	4.54

Values assume an ERW explosion height of 500 m. VITAMIN C nuclear cross sections were used. The last column gives the neutron- to gamma dose ratios at the various positions within the shield.

COMMENTARY

Computer chess and the humanization of technology

Donald Michie*

THE first serious proposal to have a machine play chess was made by Babbage¹, the British pioneer of digital computing, but was never executed. In the early years of this century the Spanish engineer Quevedo demonstrated an electro-mechanical device for mating with king and rook versus king². But it was not until the late 1940s that serious experiments with the complete game were conducted. The British logician and computation theorist Turing³, in collaboration with Champernowne and others, constructed and tested various 'paper machines' embodying mechanized strategies for chess⁴. Play was poor. In 1950 the American founder of information theory Claude Shannon⁵ published the classic paper for the theoretical ideas. During the same period de Groot's⁶ study of the thought processes of chess masters revealed that their special ability does not derive from 'computer-like' qualities of memory or of accurate, fast and sustained calculation, but from powers of conceptualization. This result has been foreshadowed by Binet's⁷ investigation in 1900 of the ability of chess masters to play many games of blindfold chess simultaneously. He concluded that in addition to *la mémoire* this accomplishment rested on *l'érudition* (the use of accumulated chess knowledge to form meaningful descriptions of positions) and *l'imagination* (ability mentally to reconstruct a position from a description). Progress has been made in mechanizing the 'computer-like' mental attributes, but little in respect of *l'érudition* and *l'imagination*.

Developments

The earliest chess programs, developed in the 1950s, have been reviewed by Samuel⁸. The modern era dates from the 1967 entry into human tournaments of the Greenblatt-Eastlake-Crocker⁹ program under the playing name MacHack. This program played at the level of a weak-to-middling club player — Category III, USCF rating 1,400–1,600 (Candidate

Master level begins at 2,000, National Master at 2,200, International Master at 2,400, Grandmaster at 2,500).

Computer chess tournaments, in which all games are program-against-program, are now organized annually in the United States by the Association for Computing Machinery (ACM). The first took place in 1970 in New York. In addition, every three years a World Computer Chess Championship sponsored by the International Federation of Information Processing Societies is held. The first¹⁰, in Stockholm in 1974, resulted in a victory for

BELLE, CHESS 4.9, NUCHESS and CRAY BLITZ, were all in the Candidate Master (2,000–2,199) range of play. The same year saw the emergence of several commercially available portable chess machines claimed by their manufacturers to rate at least 1,900 (Category I).

Computer chess has been described as the *Drosophila melanogaster* of machine intelligence. Just as Thomas Hunt Morgan and his colleagues were able to exploit the special limitations and conveniences of the *Drosophila* fruit fly to develop a methodology of genetic mapping, so the game of

Chess provides the opportunity for studying the representation of human knowledge in machines, but it took more than a century since its conception for chess playing by machines to become a reality. The World Computer Chess Championship and other computer chess tournaments where program is matched against program occur regularly. But how far can the less clever but more intelligent human master rely on the computer's brute force technology?

the program KAISSA developed in Moscow by V.L. Arlazarov, G.M. Adelson-Velskiy, A.R. Bitman and M.V. Donskoy. Chess 4.0, entered by L. Atkins and D. Slate of Northwestern University, United States, came second. Standards of play corresponded approximately to USCF 1,600–1,650. In 1975 the ACM tournament evoked play at the same general level, but produced one game, between Chess 4.4 and CHAOS, of a more distinguished standard.

In the late 1970s most progress in computer play resulted from a combination of improvements in the efficiency of deep tree-searching methods with faster speeds of available computing hardware. Chess 4.6 won the Second World Computer Chess Championship at Toronto 1977, and domination of the ACM by updated versions of CHESS continued until BELLE (Bell Labs) won in 1979. The Third World Computer Chess Championship at Linz, Austria in 1980 was also won by BELLE. The program¹¹ ran on an LSI 11/23 micro-computer linked to a hard-wired chess machine and could 'see' of the order of 100,000 board positions per second.

In 1981 the four strongest programs,

chess holds special interest for the study of the representation of human knowledge in machines. Its chief advantages are: (1) chess constitutes a fully defined and well-formalized domain; (2) the game challenges the highest levels of human intellectual capacity; (3) the challenge extends over the full range of cognitive functions such as logical calculation, rote-learning, concept-formation, analogical thinking, imagination, deductive and inductive reasoning; (4) a massive and detailed corpus of chess knowledge has accumulated over the centuries in the form of chess instructional works and commentaries; (5) a generally accepted numerical scale of performance is available in the form of the US Chess Federation and International ELO rating system.

Computational and cognitive mechanisms

The fundamental procedure, proposed independently by Turing and by Shannon, involves *lookahead* from the current position along a branching tree of possibilities. Of course, chess-masters also look ahead (concrete analysis) but on a severely restricted scale. According to de Groot, 30 positions is around the limit of

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Would the onlooker be as interested at a computer championship?

what is normally held in lookahead memory. By contrast chess programs commonly grow lookahead trees comprising millions of nodes.

Branching of the lookahead tree ranges from around 25 branches per node (if no pruning is applied) down to less than two branches per node for Masters. The number, variety and severity of pruning rules varies from one program to another. In one or two of the stronger programs all but the alpha-beta rule are effectively absent. In such a case the program seeks to make up in brute-force calculation what it lacks in selectivity. All programs apply termination rules to halt growth of the tree beyond certain limits. The main factor in termination is the occurrence of quiescent positions (Turing's 'dead' positions) in which no capture or other violent changes are in immediate prospect. At the deeper levels of the lookahead tree quiescence is taken as a sufficient indication to discontinue forward analysis.

In the Turing-Shannon scheme on completion of the lookahead tree the program applies an *evaluation function* to the terminal positions, labelling them with computed estimates of their degree of strategic strength or weakness. The labels are then *backed up* the tree by the minimax rule: that is, a node for which it is white's turn to play is credited with the maximum-valued of the labels attached to its successors, while a black-to-play node receives its label from the minimum-valued of its successors. The wave of labelling thus spreads through the tree from the terminal nodes towards the root node (representing the current position) until all its successors are labelled. The program then selects the move leading to the highest-valued of these successors, if it is white's turn to move; otherwise the move leading to the lowest-

valued successor.

The functioning of this basic mechanism can be improved by various devices aimed at eliminating redundant calculations and redundant storage. In favourable conditions the alpha-beta rule¹² for pruning almost doubles the realized depth of lookahead without altering the final result of the computation: the spirit animating this rule is that once the search has found that a particular line falls short of being selected there is no point in exploring its further ramifications to determine by just how far it falls short. Lookahead depths attained in modern computer chess lie in the range 6–15, somewhat in excess of the typical depths to which Masters and Grandmasters look (average 6–7 as found by de Groot). In spite of this the standard of computer chess remains far below Grandmaster levels. Some of the reasons are as follows:

(1) **Horizon effect.** The computer scientist and former World Correspondence Chess Champion, Hans Berliner¹³, has pointed out that reliance on the unaided Turing-Shannon procedure renders a program oblivious to all events which may occur beyond its lookahead horizon. Even though a post-horizon loss, or a post-horizon gain, may appear inevitable and obvious to the human onlooker, the program plans from hand to mouth, foolishly sacrificing material to delay a loss which cannot indefinitely be averted; alternatively it may forfeit an eventual large expectation by grabbing at a small gain.

(2) **Lack of long-range ideas.** A Master plans at the conceptual level, linking the main milestones with detailed steps as a separate operation. Contemporary

programs have no corresponding capability. In the end-game in particular, where long-range reasoning of this kind is at a premium, programs can flounder aimlessly, promoting small disconnected goals with no unifying strategic thread.

For these reasons, computer programs make a poorer showing in the end-game than in the opening and mid-game, performing like club players rather than candidate masters. Advances in sheer computer power, even micro-microsecond processors or mega-megabyte memories, are not expected in themselves materially to improve this situation. Remedies must take their departure from an appreciation of the ability of the chess-master to utilize very large bodies of highly conceptualized and cross-referenced chess knowledge. But in programs of the Turing-Shannon type the only significant repository of chess knowledge is in the evaluation function, typically a linear combination of terms measuring such features as material advantage (conventional scores: 9 for Q, 5 for R, 3 for B, 3 for N, 1 for P), king safety, piece mobility, pawn structure, rook control of files and so on. Typically the number of features contributing terms to the evaluation function in state-of-the-art tournament programs lies in the range 30–50.

So simple a scheme is too weak to represent the fine structure of human knowledge marshalled in the standard expository works such as Reuben Fine's *Basic Chess Endings*. Contemporary research is directed towards buttressing the Turing-Shannon paradigm along a line sometimes described as the 'knowledge approach'. Essential to this approach is the extension of studies like Binet's and de Groot's to the discovery of the basic concepts (properties and relations of pieces, files, ranks and so on which the Master uses as the bricks and mortar of his mental descriptions). Chase and Simon¹⁴ found that the relations of defence of one piece by another, proximity of pieces, and being of the same denomination or colour were all used as mental building-blocks, and that a particularly important relation for binding together clusters of pieces held as a unit in memory was combination of pieces of the same colour to converge on the opponent's king position.

Tan¹⁵ formalized the process of conceptualization for the special case of pawn structures. His computer program was able to describe pawns-only positions in terms of 'islands' and 'fronts' forming pawn-relations graphs, from which 'attack-defence-diagrams' are automatically constructed. The dynamic potentialities of the position are thus summarized. More recently a computer induction algorithm¹⁶ derived from Hunt's 'Concept Learning System'¹⁷ has been used to synthesize complete machine-executable theories for the endings king and pawn versus king¹⁸ and king and pawn (on a7) versus king and rook¹⁹.

Chess is a two-person finite game with perfect information which satisfies the zero-sum condition — an outcome which is good for one player is bad in equal measure for the other. For any such game it is theoretically possible exhaustively to calculate backwards from the terminal (checkmate or drawn) positions in such a way as to determine for every position whether it is drawn, won or lost and in the latter two cases what continuations correspond to best strategy. In practice such computations, even if performed on the fastest conceivable computers, are infeasible except for end-games simple enough to contain less than a billion or so legal positions. Such computations were first done²⁰ for elementary endings such as king and rook versus king (KRK) and king and pawn versus king (KPK) which consist respectively of 50,015 and 179,656 legal positions. They have been extended to all the non-trivial four-piece endings, such as KQKR, KRKN and so on and to a subset of KPKP (M.R.B. Clarke, personal communication). The most complex enumerations to have been performed in this way are KQPKQ²¹ and KRPKR²², of which the first is notable for having been consulted with good effect by Bronstein during adjournment of a master tournament in Kiev. Significant findings have been made with the use of these end-game 'databases', including the previously unsuspected prevalence of serious error in master texts on the end-game. Thus Fig. 1 shows a derivative of the celebrated position given by al-Adli in the ninth century, re-discovered and (incorrectly) analysed in *The Chess-Players' Chronicle* in 1859, and repeatedly (and incorrectly) re-analysed since then. Among errors of Grandmaster Fine's analysis in *Basic Chess Endings* is his classification of the position in Fig. 1 as a draw. Computer analysis shows that knight-capture or mate can be forced in 12 further moves²³.

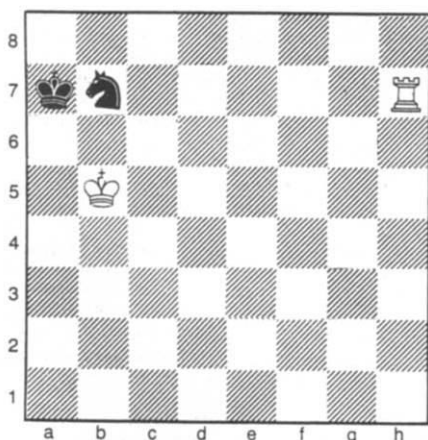


Fig. 1 One of two positions generated in Fine's analysis of the al-Adli position (see text) to which the wrong won/drawn status is assigned. Fine states that, after black's Kb8, white can only draw. Computer analysis reveals that by Kc6 white can then win in 12 moves. As shown in ref. 23, lesser errors abound.

Brute-force computing in technology

The available power of computation advances at the rate of almost tenfold every five years. For today's large machines one hundred million calculations per second is not abnormal. Measured on a comparable scale, the human brain musters perhaps five per second. The brain, being able to deploy a large associative store of pattern-based concepts, is not normally used in this restricted sequential way. Otherwise feats such as recognizing a person from a photograph, or understanding his speech, would be impossible for a device with such weak calculational powers. On the other hand computing systems still rely primarily on brute force. So long as the present rate of advance in hardware technology continues, they can afford to. But can we, their less clever but more intelligent masters, afford to let them?

Several recent incidents have involved complex computer control systems. The suggestion is that reliance on the escalating power of brute force may be heading towards danger. However effective and reliable such systems may be in normal conditions, use of brute force may not be worth the price paid during the rare episodes when a computer-controlled power station or military installation or air-traffic control system malfunctions. On these occasions a new factor becomes paramount: the human operator or supervisor needs to follow what the computing system 'thinks it is doing'.

The computer's processes are measured in millions of steps per second. The human's proceed very slowly — but in a richly furnished space of descriptive concepts. These concepts are not mirrored in any way in the machine's relatively small memory. So when operating conditions stray from the norm, useful dialogue between the two breaks down. In its report on the Three Mile Island accident the Kemeny Commission concluded that the main failures were operator failures, and that the main cause of operator failure was bewilderment by the stream of messages, warning displays and the like from the control computer²⁴.

If such unsettling phenomena deserve laboratory analysis, then we could hardly find better material than the game of chess. The current world computer chess champion examines a tree of more than ten million possibilities in lookahead analysis before selecting a move, and is able on this basis to stand up to Grandmasters in purely tactical play. The Grandmasters, by virtue of their associative stores of conceptualized chess knowledge, have the edge in strategic, or positional play. But as earlier stated a Grandmaster's mental investigation of lookahead positions averages at most 30. So the kind of mismatch that was noted by the Kemeny Commission, namely between the calculation-rich but concept-poor computer and the calculation-poor but

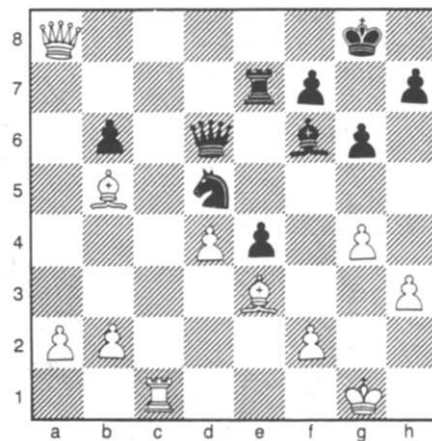


Fig. 2 Position in the Toronto game DUCHESS against KAISSA after white had played Qa8+. Black's play of the apparently meaningless rook sacrifice Re8 was seen by an audience which included several strong chess-masters as a grotesque blunder. Overnight analysis by KAISSA's programmers showed otherwise.

concept-rich human, should be reproducible in the computer chess laboratory. This is indeed the case, as has already been shown through an analysis of the mechanisms employed in state-of-the-art computer chess and its theoretical basis²⁵.

Machine penetration

Machine penetration into complex positions began to reach beyond the human horizon as early as 1977. In the Second World Computer Chess Championship held that year in Toronto, the position shown in Fig. 2 arose in a game between the then reigning champion KAISSA, a Moscow program running on an IBM 168 computer, and the North Carolina program DUCHESS. DUCHESS had just given check with the queen. To the several hundred computer scientists and chess players in the auditorium the only reasonable option was to move the king out of check. KAISSA instead interposed a rook, promptly losing to QxR check. With a whole rook advantage DUCHESS had no difficulty in crushing KAISSA in another 15 or so moves, and the Russian team handed in KAISSA's resignation in the conviction that they had been robbed by an unsuspected program error.

Next morning Arlazarov and Donskoy announced the result of a retrace operation which had occupied them half the night and had revealed no evidence of error. On the contrary, deep-going scrutiny showed KAISSA's apparent blunder to have been a brilliancy which purchased an extended lease of life for a program which had already arrived in a hopelessly lost position. The rook sacrifice had cleverly averted a mating combination which both KAISSA's and DUCHESS's lookaheads were deep enough to spot, but which eluded onlookers who included former world champion Grandmaster Mikhail Botvinnik.

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Expert versus martian systems

Now consider chess as a laboratory model of real-life decision-taking. Imagine KAISSA's brute-force computations to be those of an automated control system for a nuclear power station. Let Grandmaster Botvinnik be the engineering supervisor, highly knowledgeable about the domain, assigned to monitor the system's decisions and to intervene with manual over-ride when he judges malfunction to have occurred. The machine makes an unexpected decision. Does the supervisor intervene? Lacking the calculational power fully to probe the system's martian mentality, let us suppose that he does. Disaster follows. Yet had he been able to interrogate the system he would have realized that the seemingly aberrant action was really geared to buying vital time — time in which staff could be evacuated, population warned, ambulances and fire engines summoned and so forth. Yet he has to decide on the basis of his knowledge and best judgement. Not being a martian, he decides wrongly.

This problem cannot be brushed aside by improvements to the program's surface features, such as better trace and diagnostics and more 'user-friendly' command languages. For the particular case, chosen from 1977 vintage computer chess, this might suffice. But as the depth of calculation increases, a point is reached at which mere surface modifications will not do. Radical reconstruction of the program becomes necessary, using as building-blocks machine representations for the very same concepts as those which the knowledgeable human brings to bear on the problem.

This approach leads to a new form of computing system, known as the 'expert

system', which is deliberately built in the human rather than martian mental mould. The use of such systems to act as interpretative buffers between the two mentalities was first demonstrated at the Rand Corporation in RITA, a computer program knowledgeable both about the intricacies of the ARPA trans-continental computer network and about the limitations of non-programming personnel desirous of using the network²⁶.

Brute-force computing is pushing information technology towards regions of complexity which only machines will be able to penetrate. To make it possible for them to report back what they see, RITA-like developments in human-interface software will be required. An eight-year programme of research and development in advanced computing technology recently announced by the Japan Information Processing Development Center is based in part on this conception, namely on the design and construction of intelligent knowledge-based systems²⁷. Their role will be to mediate between the world of machines and the world of people.

Future work and prospects

Large-scale transfer of human knowledge from books (or brains) to computers has not been achieved in any human intellectual domain. Computer chess is at the leading edge of experimental attempts to achieve it. Endeavours centre round three foci:

(1) The design of data-structures in forms suitable for representing conceptualized knowledge (descriptions, patterns and theories) which are also convenient for the human user to modify and increment interactively.

(2) Improved facilities for inductive

inference, so that programs can acquire new knowledge both from illustrative examples supplied by human tutors, and also from the results of their own internal generation of examples for self-administration.

(3) The engineering of conceptual interfaces between program and human expert, making it easier for the latter to 'teach' the machine.

Advances under all of the above headings are required before the goal of Grandmaster play by machine can be seriously envisaged. By the same token, few investigators doubt the ultimate attainment during the present decade of Grandmaster levels. Apart from benefits to the arts of programming, such an extension of technique also has a bearing on the study of cognition.

I thank Senior Master Danny Kopec for helpful comments.

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NEWS AND VIEWS

Twelve wise men at the Vatican

from J.M. Lowenstein

A RECENT meeting* of twelve scholars at the Pontifical Academy of Sciences in the Vatican Gardens proved historic in two respects. First, the group, which consisted of palaeontologists, geneticists and molecular biologists and which had been specifically assembled to reconcile the palaeontological evidence of primate evolution with that from molecular biology, was able to agree on a scenario for primate evolution over the past 30 million years. To many protagonists of the two disciplines this will seem something of a miracle. Second, the highest scientific body of the Catholic Church produced a strong statement supporting the evolutionary hypothesis as the explanation for the origin and diversity of living primates — just a few weeks after the hundredth anniversary of Darwin's death.

Presiding over the working group was the President of the Pontifical Academy, Carlos Chagas, a neurophysiologist from Brazil and scientific adviser to Pope John Paul II. The pope reportedly takes a keen interest in the activities of the Academy.

Palaeontologists and molecular biologists have been divided over whether the various living apes and man diverged early on (20 Myr BP) or late (5–7 Myr BP). Some fossil evidence, particularly the dating of *Ramapithecus*, considered by many palaeoanthropologists to be hominid, supports the earlier date. A mass of biochemical data showing about 99 per cent identity between the DNA and proteins of chimpanzee, gorilla and man implies, from inferred rates of DNA and protein change, that the separation occurred at the later date.

The working group was able to reconcile the fossil and biochemical evidence by taking into account evidence that *Ramapithecus* is not hominid but is more probably related (along with *Sivapithecus*) to the orang-utan lineage. Particularly important are the recent discovery of a *Sivapithecus* face which has many orang-

utan-like features (see Pilbeam *Nature* 295, 232; Andrews *News and Views* 295, 185; 1982) and the finding that limb bones attributed to *Ramapithecus* or *Sivapithecus* (but not found in association with jaws and teeth) strongly suggest an arboreal adaptation. Supporting evidence comes from a new radioimmunoassay technique. Preliminary results (Lowenstein) show the fossil proteins of *Ramapithecus/Sivapithecus* to be most like the serum proteins of orang-utan, gibbon and gorilla, less like those of man and chimpanzee and least like those of monkey and other mammals — suggesting the Miocene genera *Ramapithecus/Sivapithecus* were hominoid (ancestral to apes or to apes and humans) rather than hominid.

The first true hominids were agreed to be the australopithecines of East and South Africa, dated from about 4 Myr BP. Coppens, who with Johanson and White named the species *Australopithecus afarensis*, now considers that the observed large variation in size may not be due to sexual dimorphism as previously thought, but may indicate that more than one species was present at that early time. In the period between 4 and 14 Myr, when the common ancestor of man, chimpanzee and gorilla probably lived, there is no significant African fossil record of hominids or hominoids and no fossil record at all for chimpanzee and gorillas. Fossils from this period are badly needed to decide the path of human evolution.

The earliest record of monkey-like and ape-like primates, known from the Fayum formation of Egypt, was reviewed by Simons, who also described a new genus, *Qatrania*. *Aegyptopithecus*, earlier discovered in the Fayum by Simons, has teeth resembling those of apes but limb bones resembling those of New World howling monkeys. *Aegyptopithecus*, dated at 25–35 Myr, may be ancestral either to Old World monkeys and hominoids or to hominoids only, depending on the dating of the monkey-hominoid split. Molecular data suggest the split occurred 20–25 Myr BP which would make *Aegyptopithecus*

ancestral to both groups. Palaeoanthropologists commonly date the split much earlier, so that in their view, *Aegyptopithecus* is a hominoid ancestor only. Here, as in so much of primate evolution, accurate dating of divergences between lineages becomes critical.

One characteristic proposed as evidence of a phylogenetic relationship between *Ramapithecus* and *Australopithecus* is the presence of thickly enamelled molars. In Greenfield's opinion, this trait has very limited phylogenetic significance and does not support the inferred relationship. Rather, bipedalism and not small canines or an enlarged brain appears to be the emergent hominid trait — although what ecological conditions encouraged bipedalism is not at all clear.

Molecular biology can be expected to prove of increasing value in providing information about the relationships of extant higher primates. Doolittle pointed out that the study of pseudogenes, which change rapidly without any apparent selection pressures to restrict base pair substitutions, may provide particularly useful data.

Many other controversial areas of primate evolution were discussed. Tobias put forward the view that *Homo habilis* may have been capable of speech (albeit rudimentary) on the basis of an inferred well-developed vocal tract, endocast evidence of possible Broca's and Wernicke's areas, and a stone tool technology reflecting learned technical skills requiring speech for their transmission.

Overall, though, the working group was able to agree on its conclusion:

"We freely acknowledge that there is room for differences of opinion on such problems as species formation and the mechanisms of evolutionary change. Nonetheless, we are convinced that masses of evidence render the application of the concept of evolution to man and other primates beyond serious dispute". □

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*Participants at the conference, held 24–27 May 1982, were E. Bone (Belgium), Y. Coppens and J. Lejeune (France), R. Doolittle, L. Greenfield, J. Lowenstein, D. Pilbeam and E. Simons (USA), C. Pavan (Brazil), G. Sermoniti (Italy) and P. Tobias (S. Africa).

Beneficial autoimmunity?

from Charlie Janeway

THE immune system has a difficult part to play in the biology of vertebrate species for it must recognize and respond to a great variety of microorganisms which have a short generation time and can evolve more rapidly than the animals they invade. The immune system appears to have solved this problem by a general strategy of generating and amplifying the expression of those members of the very large set of recognition molecules or antibodies that are suitable for binding the potentially pathogenic organisms in its environment. At the same time, a complex series of regulatory mechanisms has been developed to prevent autoimmunity, the activation of such responses towards constituents of self. One type of autoimmune response, however, may have a central role in immune regulation: since each specific antibody molecule has a chemically and hence antigenically unique structure, recognition of that structure by the immune system could be used to control immune responses¹. Whether it does indeed have such a role is a matter of contention. A paper in this week's issue of *Nature* (p.447) provides an important piece of evidence supporting the existence of this form of autoimmune response and its potential regulatory function².

The general term for an antigenic determinant borne on the portion of the antibody molecule involved in specific antigen binding is an idiotype: the collection of idiotypes on a molecule make up its idiotype. Since most idiotypes are present at very low levels in normal serum, it appears that animals are not tolerant of these idiotypes, as they are of other serum protein antigens present at far higher concentrations. When an animal is immunized, the concentration of the idiotypes carried on the antibodies specific for the immunizing antigen undergoes a dramatic increase. In some cases, it has also been possible to demonstrate directly that the increase in those idiotypes is also accompanied by production of antibodies against them (anti-idiotype antibody)³. This occurs at a time at which the antibody

response is being reduced, and, since injection of anti-idiotype antibody is also suppressive of specific antibody responses⁴, the logical conclusion has been that the anti-idiotype antibody has an immunoregulatory role. Over the past few years, similar observations have been made in several experimental systems and have frequently employed monoclonal idiotypes and monoclonal anti-idiotypes as probes for regulation⁵. In addition, anti-(anti-idiotype) and anti-(anti-anti-idiotype) antibodies have been produced by artificial immunization schemes, and shown to affect the expression of idiotype in antibody responses⁶. It is thus clear that animals are able to make such responses, and that such antibodies, once produced, do affect the expression of antigenically related immunoglobulins.

What Pollok *et al.*² have shown is that, during the course of a normal immune response to a bacterial antigen, phosphorylcholine (PC), anti-(anti-idiotype)-specific B lymphocytes are activated, as demonstrated by making hybridomas that secrete monoclonal antibodies of this specificity from spleen cells of immunized mice. Furthermore, when the monoclonal antibody produced is injected into syngeneic mice before immunization with PC, it profoundly alters the expression of idiotype and of anti-PC antibody. While such an observation falls short of demonstrating an actual regulatory role for such auto-anti-(anti-idiotype) antibodies, it is certainly consistent with such a role and, since the antibodies were induced by immunization with antigen (PC) with which the anti-(anti-idiotype) antibodies do not react, it suggests that some intervening anti-idiotype response must also occur.

A number of questions are left unanswered by these studies. Some are technical, but important: while the anti-(anti-idiotype) antibodies do not react with PC, the immunization was actually performed with intact bacteria; do the putative anti-(anti-idiotype) antibodies react with the vaccine? If so, they would represent another example of the sharing of idiotope by two antibodies directed at distinct determinants on an antigenic particle^{7,8}. More

important, one would not, then, need to postulate anti-idiotype as the *sole* stimulus for production of the putative anti-(anti-idiotype) antibody. A second question needs further examination: is the anti-(anti-idiotype) actually secreted during this response, or has the fusion itself allowed expression of a B cell product that is normally not secreted? It is not known whether B cells giving rise to hybridomas are actually in a secretory or pre-secretory phase of activation when fusion occurs.

If one allows that the experiments of Pollok *et al.* demonstrate the presence of a *bona fide* auto-anti-(anti-idiotype) response, and that the antibody produced does play a part in normal regulation, several obvious questions need to be answered. First, how is anti-(anti-idiotype) antibody production induced by an antigen with which the anti-(anti-idiotype) does not react? Many types of anti-idiotypic elements have been described: B cells², two distinct classes of helper T cells^{9,10} and various cells in suppressive pathways¹¹⁻¹⁴. Which, if any, of these anti-idiotype cells or molecules activate production of anti-(anti-idiotype) needs to be determined; a first choice might be the anti-idiotypic helper T cell described by Cerny and Caulfield¹⁰ which appears to act directly on idiotypic B cells in the absence of antigen, although activation of the B cell by anti-idiotypic antibody may also be required¹⁵.

Second, and equally important, what cell is the target of the anti-(anti-idiotype) antibody in modulating expression of the original idiotype? Again, the same cell types may be involved, since anti-(anti-idiotype) antibody presumably affects anti-idiotypic cells or molecules. However, since the effect in the present case is suppression, the logical choice is either direct suppression of an anti-idiotype helper by anti-(anti-idiotype), or activation of a suppressor T cell that is anti-idiotypic, as demonstrated by Owen and Nisonoff¹¹ in the response to azophenylarsonate.

A major difficulty confronting advocates of anti-idiotypic immunoregulation is demonstrating the importance of such



Schematic representation of the experiment of Pollok *et al.* Immunization with a phosphorylcholine-bearing bacterial vaccine induces production of antibody marked by a particular idiotype. B cells from these mice, isolated as monoclonal antibody-secreting hybridomas, produce an antibody reacting with anti-idiotype, but not binding to PC. The authors propose that production of this antibody is stimulated by an anti-idiotype, here represented as anti-idiotype antibody. This anti-(anti-idiotype) can also feedback, again presumably via an anti-idiotype antibody or cell, to prevent production of anti-PC bearing the original idiotype.

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effects during immune responses *in vivo*. Previous experiments have often employed heterogeneous antisera raised by vigorous immunization regimes. The studies of Pollok *et al.* avoid this difficulty, but do not answer the quantitative question of whether the responses are crucial to the biology of the immune system, or whether they represent largely an experimental curiosity. The extraordinary capabilities of

the immune system to produce antibodies of virtually any specificity, as well as the heterogeneity of these antibodies, make this question enormously difficult to approach, let alone to answer. The finding of one element of a putative idiotypic regulatory network as a result of immunization with antigen is, however, a further incentive to pursue this line of investigation to its conclusion. □

Zero-momentum atoms in superfluid helium-4

from P. V. E. McClintock

ONE of the many oddities of liquid helium is that it often seems to behave as though it were a gas. It does not, of course, expand to occupy all the available space in its container — it could not be described as a liquid at all, if this were the case — but in terms, for example, of its very low density, its refractive index close to unity, its feeble interatomic forces, its tiny surface tension and its extremely small viscosity (less than that of air at room temperature, even for normal, non-superfluid helium-4), its properties often seem more gas-like than liquid-like.

The natural question to ask, therefore, is just how far this analogy can be taken. In particular, given that helium remains fluid down to the lowest attainable temperatures, does it fall into the sort of exotic ground state predicted for ideal gases at very low temperatures? For 'real' gases, of course, the question does not arise, because they would no longer be gaseous at the low temperatures in question; but for liquid helium . . . ? These questions, first posed more than four decades ago by F. London (*Nature* 141, 643; 1938), have remained central to any fundamental discussion of the liquid heliums ever since. It has, in fact, been known for some time that the profound differences which exist between the low-temperature properties of liquid helium-3 (the rare isotope) and those of liquid helium-4 can mostly be accounted for quite well if the two liquids are considered by analogy with gases. What has remained extremely uncertain, however, is whether or not a finite proportion of the atoms in liquid helium-4 exist in the peculiar zero-momentum state (so-called Bose-Einstein condensate) which is predicted for an ideal gas in the same conditions. It is this crucial question which seems now to have been answered as a result of work at Chalk River, Canada, by Sears, Svensson, Martel and Woods, published in a recent issue of *Physical*

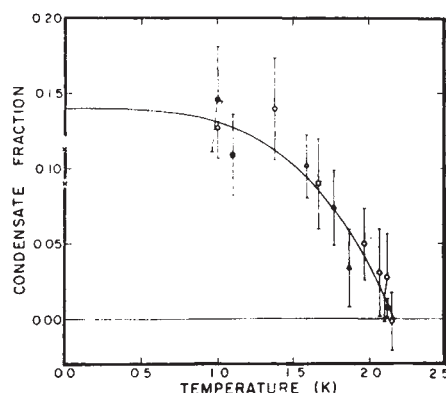
Review Letters (49, 279; 1982).

Bose-Einstein condensation represents one of the most intriguing predictions of quantum-statistical mechanics. The phenomenon is expected to occur in a gas which (1) consists of bosons (made from an even number of fundamental particles) and (2) is ideal (point particles with zero interatomic forces). Helium-4 clearly satisfies criterion (1) because the atom consists of two protons, two neutrons and two electrons; but the same cannot be said of criterion (2). There has been a continuing debate about the extent to which the existence of the interatomic forces, albeit very weak ones in the case of liquid helium, would modify the behaviour of the assembly. In an ideal gas, it is expected that there will exist a critical temperature, T_c , below which atoms will start to condense into the zero-momentum state. The de Broglie wavelength of atoms in this state is infinite (in practice, equal to the dimensions of the container) so that the more familiar concept of atoms as 'particles' becomes, to say the least, a little strained. The onset of the condensation phenomenon is predicted to occur with extreme suddenness as the temperature is lowered. It turns out that, as T falls below T_c , the requirements of quantum statistics can be satisfied only if some of the atoms are 'forced' into the zero-momentum ground state. As a result, the occupation number of this state should suddenly start to shoot up from a quite negligible value above T_c to a finite value as T falls below T_c ; and it should approach unity for $T \ll T_c$, corresponding to almost all the atoms in the assembly being in the zero-momentum state. The existence of interatomic forces is expected to modify this picture to a considerable extent, however. In particular, it can be shown that the condensate fraction at $T=0$ should be reduced to a value lower than the 100 per cent characteristic of an ideal gas.

One of the great attractions of applying these ideas to liquid helium is that the superfluid transition, observed at the so-called lambda point of $T_\lambda = 2.2\text{K}$, may then

be identified with the onset of Bose-Einstein condensation — albeit in a modified form because of the interatomic forces. This identification is supported by the facts that the superfluid transition occurs very suddenly, just as predicted for Bose-Einstein condensation; that it takes place at approximately the calculated temperature; and that many of the strange properties exhibited by liquid helium below T_λ , for example its quantized rotational modes, can readily be accounted for if it is assumed that some condensate is present. The variation of the specific heat with T near T_λ and many other properties of the liquid are, however, entirely different from those predicted for an ideal Bose-Einstein gas. Thus, although all such discrepancies may be attributed — reasonably plausibly — to the influence of the interatomic forces, there has remained a nagging doubt as to whether the liquid really does have a finite condensate fraction or not.

Attempts to test London's ideas through a direct determination of the magnitude of the condensate fraction have been based on neutron and X-ray scattering from the liquid. For technical reasons, both the experiments and their interpretation have been rather difficult. Part of the problem arises from the fact that the condensate fraction is evidently quite small, thus necessitating a measurement of relatively high precision. Some of the earlier experimental results were, in fact, quite consistent with the condensate fraction being zero, a most discouraging conclusion. There has also been some degree of controversy about the validity of one of the (apparently) most successful techniques which has been used, based on measuring the temperature dependence of the two-



The condensate fraction in liquid helium-4 plotted as a function of temperature (from V.F. Sears, E.C. Svensson, P. Martel & A.D.B. Woods *Phys. Rev. Lett.* 49, 279; 1982). The filled symbols represent new measurements based on inelastic neutron scattering. The open symbols correspond to values determined from the measured temperature dependence of the two-particle correlation function, including some earlier measurements (squares) by H. N. Robkoff, D. A. Ewan & R. B. Hallock (*Phys. Rev. Lett.* 43, 2066; 1979). X represents theoretical predictions for $T=0$.

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particle correlation function $g(r)$.

The new results now presented by the Chalk River group, shown by the filled symbols in the figure, are based on inelastic neutron scattering, a completely different technique the validity of which has not been impugned. The open symbols represent data derived from the temperature dependence of $g(r)$. Agreement between results obtained by these two techniques would appear to be excellent and there is really no longer any room for serious doubt as to the existence of a Bose-

Einstein condensate in liquid helium-4 below T_λ . It appears that the magnitude of the condensate fraction rises from zero at T_λ and approaches a limiting low-temperature value of about 14 per cent below 1 K, a value which is in quite reasonable accord with recent theoretical predictions.

The principal conclusion, that London's imaginative suggestions of 44 years ago are indeed correct, will provide a source of considerable satisfaction for all those with an interest in liquid helium. □

Changes at the crust-mantle boundary

from Jack Oliver

MANY recent observations indicate that the current view of the Moho, the crust-mantle boundary, needs revision. In particular, the widely held concept of the Moho as a sharp and laterally uniform boundary appears misleading in its simplicity, and may even be a major barrier to a better understanding of the nature and evolution of continents.

A brief look at the history of ideas about the Moho reveals how concepts can sometimes facilitate, and sometimes inhibit, understanding of the Earth. The Moho was discovered by the Serbian seismologist A. Mohorovičić in 1909. His evidence, seismic waves generated by nearby earthquakes and refracted to the surface, indicated a zone of rapid increase of seismic velocity at depth. Since then, innumerable seismic refraction surveys have shown that the velocity increase is nearly always found in continental areas at depths of 30 to 50 km and in ocean basins at depths of perhaps 10 to 12 km.

Until recently, the only method used for analysing seismic refraction data was, in essence, that employed by Mohorovičić. The method, based on ray theory and the time-distance plot, is biased strongly towards abrupt discontinuities and plane-bounded layers. Although the biases are well known to seismologists, results are often presented to others in a way that inadvertently overemphasizes these characteristics.

At some point, Mohorovičić's zone of rapid velocity increase became the crust-mantle boundary. The attractions of this step were many. The velocity increase could be readily observed. It implied a major density increase and hence a possible place for isostatic compensation and for the sources of certain gravity anomalies. It could conveniently be assigned to mark the boundary between acidic and basic rocks

of the crust and the ultrabasics of the mantle.

Some awkward scientific and semantic problems arose from this step, but they aroused little attention. For example, it was first thought that lateral variations were absent below the Moho. However, as mantle thickness is different under continents and oceans, the velocities and the velocity gradients cannot be identical. Later, plate tectonics called for, and observations demonstrated, a more dynamic and heterogeneous mantle.

The simple concept of the Moho has been so appealing and so emphasized that it may have become repressive. Sometimes sub-Moho rocks with velocities of 8.4 km s⁻¹ at one site have been lumped as 'mantle' with sub-Moho rocks at 7.6 km s⁻¹ from another site without attracting much attention to the lateral differences. A change of this magnitude in the vertical direction could form a spectacular 'low-velocity layer' and command astonishment and imaginative interpretation! The concept of a uniform mantle has been so ingrained that some have risked circular reasoning and interpreted all variations from the standard 8.1 km s⁻¹ as effects of anything but composition.

Now, new kinds of observations, and reconsidered old ones, are demonstrating that much more is to be learned about the Moho. In a recent paper, Hale and Thompson¹, following the lead of Fuchs² and Meissner^{3,4}, draw attention to the complexity of the boundary zone in the vertical dimension. They describe a laminated zone several kilometres thick revealed at five sites by recent seismic reflection profiling, noting only minor lateral variations at any one site and similarity among all five sites. The laminated structures are variously interpreted as cumulates, metasediments, tectonic banding, or lenses of partial melt. Other reflection studies, such as the BIRPS profile on the shelf north-east of Britain,

indicate a simpler boundary zone at that locality, but one with evidence for a fault that may be traced through the entire crust to the surface. Three COCORP reflection lines crossing the Quachita-Appalachian orogen in the USA suggest that modern continental Moho to the seaward ends of the lines may, in fact, be former oceanic Moho buried during major thrusting. Or perhaps Moho is the base of the former oceanic sedimentary layer now overlying oceanic crust eclogitized to display mantle properties.

Other geophysical techniques are providing different types of evidence on Moho. Studies of the earthquake-generated phases Pn and Sn reveal major lateral variations in velocity and attenuation in the uppermost mantle. In some places, such as the shallow mantle of the Atlantic and the Pacific and the down-going slab of subduction zones, this zone enigmatically forms perhaps the most efficient transmitter of high-frequency seismic waves within the Earth; in others it is a severe attenuator.

Gravity data are being used in conjunction with seismic data to explore lateral variations in the Moho⁵, and new kinds of computer handling of these data will facilitate this approach^{6,7}. Drilling seems limited to depths of 15 km or less for the near future and hence could penetrate Moho only in anomalous places or in the ocean basins.

Seismic refraction studies have long shown spatial variations in sub-Moho velocities that could be, but rarely have been, interpreted geologically. The Soviet DSS method uses refractions and wide-angle reflections in a particularly comprehensive fashion, and important new methods of analysis of refraction data, such as the τ/p method and ray tracing through arbitrary structures, are being developed to minimize bias to special geometries and to facilitate incorporation of geological information and concepts.

The Moho at sea has been best observed by seismic refraction measurements, but occasionally vertical reflections are obtained⁸ and improved techniques may produce more such information. Geochemical studies of igneous rocks indicate consistent lateral variations of the source rocks in the mantle⁹.

In the transition zone between continents and oceans, observations of Moho are characteristically difficult, and the structure of the mantle there is usually derived by inference. This situation is unfortunate because some evidence, such as the general uniformity of thickness of the continental crust, suggests substantial ductile flow near the base of the crust, whereas the inferred configuration of the transition zones is sufficiently abrupt to suggest that such flow is resisted.

Perhaps best of all, former Moho can now be seen, touched and sampled. Within the last 15 years, many pieces of former oceanic Moho have been re-

cognized on land as parts of ophiolite sequences that have been moved onto continents as oceans closed¹⁰. The boundary zone between the gabbros of the oceanic crust and the peridotite overlying harzburgite of the mantle is evident and informative about the generation of lithosphere at oceanic spreading centres.

Furthermore, as shown by Berkhemer¹¹, the former continental Moho and associated deep crustal rocks are exposed in the Ivrea zone of the Alps, and, in a recent paper, Fountain and Salisbury¹² list areas on four continents where former continental Moho may be at or near the surface. In general, the rocks from near the Moho have a layered structure that is much more complex than most geophysical observations suggest, and they have a special attraction for detailed study at this time of increasing emphasis on understanding the deep continental crust.

Within the last few decades, the name of the boundary under discussion has grown simpler, going from 'Mohorovičić discontinuity' to 'Moho' to, sometimes, 'M'. Our knowledge of the boundary itself is moving in the opposite direction. □

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Transgenic organisms and development

from William Petri

DEVELOPMENTAL biologists have claimed that the secret of cell differentiation in higher organisms is differential gene expression. Unfortunately, documentation of the molecular mechanisms involved has been sparse and superficial and lagged far behind that for prokaryotic systems such as *lac* and λ . However, the mood at a recent meeting on molecular aspects of development* was optimistic that real progress towards understanding the control of gene expression during development may be at hand. Much of the progress will be due to the application of the recombinant DNA technologies and the recently acquired ability to create completely¹ transgenic organisms (organisms all of whose cells contain a copy of an experimentally introduced DNA).

In a transgenic organism, a copy of an experimental gene is inserted very early in development; ideally the gene will be integrated into the germ line. If this occurs, progeny will be produced that will carry the foreign gene in every cell, and the expression of the gene can be assayed throughout development in every tissue. Comparisons among organisms containing systematically modified variants of the same gene and nucleotide sequences associated with it (for example, ref. 2) should allow identification of the genetic elements responsible for controlling tissue-specific and developmental stage-specific

gene expression. Such elements are the core of developmental regulation, and their elucidation has not been possible using the previously available test systems involving cell-free transcription, injection of amphibian oocytes and transfection of cultured cells since they are primarily limited to investigation of cellular and genetic factors necessary for gene expression *per se* or those influencing transcriptional efficiency. Hence, completely transgenic organisms should form the basis of a boom in our understanding of developmental control mechanisms. Fortunately, such organisms are now not only possible but apparently more easily obtained than expected.

B. Mintz (Institute for Cancer Research) reported two routes of entry for cloned DNAs (in particular, a plasmid containing both the human β -globin and herpes simplex virus thymidine kinase genes) into the mouse germ line: egg pronuclear injection and transfection of totipotent teratocarcinoma cells followed by blastocyst implantation. Both methods succeeded at experimentally usable frequencies (up to 20 per cent in preliminary experiments). However, expression of the HSV-TK gene was observed in only one of five parental transgenic mice³. Importantly, the introduction of exogenous DNA via a recently developed totipotent mouse euploid teratocarcinoma culture cell line will allow pre-selection and pre-characterization of genetic changes before re-introduction into the mouse.

Using microinjection into mouse eggs, R. Palmiter (University of Washington)

reported successful non-homologous integration of cloned fusion genes (5' mouse metallothionein-I promoter and 3' HSV-TK structural gene) into the germ line⁴. In most transgenic mice capable of expressing the HSV-TK activity, the gene was inducible by heavy metals, the normal inducers of metallothionein, indicating the successful introduction of functional controlling elements into recipient mice. Fusion gene expression in induced progeny is, however, highly variable, often extinguished, and unrelated to parental levels and to gene dosage. The unpredictable expression of introduced genes seen in both groups may hinder the usefulness of transgenic mice as assay systems for developmental control elements; however, it may also provide insights into the ways gene expression can be modulated or extinguished.

Fortunately these problems may not plague all transgenic systems. G. Rubin (Carnegie Institute), in collaboration with A. Spradling, reported the successful germ-line incorporation of cloned *rosy*⁺ (xanthine dehydrogenase) into *rosy*⁻ *Drosophila* by injection into the poleplasm of fertilized eggs using the P element responsible for hybrid dysgenesis as a vehicle. A very high proportion of fertile adults resulting from injected *rosy*⁻ eggs produced *rosy*⁺ progeny (20–50 per cent), and *rosy*⁺ segregants always contain extra *rosy* material whereas *rosy*⁻ segregants do not. Hence, in contrast to the mouse results, here it seems that at least some incorporated genes are expressed in each transgenic line. Although integration of the injected *rosy*⁺ genes is non-homologous, two features of the system are intriguing. First, six of twelve *rosy*⁺ insertion sites are very close to the normal *rosy* locus and second, integration occurs with the precise exclusion of the plasmid cloning vector. Overall, transgenic *Drosophila* appear to provide a promising system for the study of developmental control elements and effects of gene location on expression.

The key difficulty of where and how important changes in gene activity occur in the long and increasingly complicated process of development received much attention (and see refs 5 & 6, for example). Selected points brought out at the meeting follow.

Contrary to previous notions, few genes seem to turn on sharply in early development. Most sequences prevalent in the pluteus of the sea urchin (E. Davidson, Caltech)⁷ or the gastrula of *Xenopus* (I. Dawid, NIH) are already present in maternal RNA. Yet these sequences do not simply represent housekeeping functions since most are not found in adults (Davidson). Generally, an important level of control in early development seems to be variable turnover rates of cytoplasmic

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transcripts (Davidson)⁸. Curiously, a major portion of the egg cytoplasmic poly(A)-containing RNA in sea urchins and in *Xenopus* contains interspersed repeat sequences in relatively long transcripts reminiscent of nuclear RNA (Davidson, Dawid)⁹. They could result from a failure of termination, as seems to be the case in the unusually long transcription units characteristic of lampbrush chromosomes (J. Gall, Yale University)¹⁰, or perhaps from lack of intron splicing (Davidson). The role of these molecules, if any, remains speculative. It is unlikely that they are translated without further processing since many of the repeated elements contain multiple stop codons (Davidson); hence, it is possible a type of developmental control could reside in tissue- and stage-specific completion of RNA processing.

Genetic transcriptional control elements are known to exist upstream from the TATA box in cases such as dimethyl sulphoxide induction of β -globin (Maniatis, Harvard University) and heavy-metal induction of metallothionein (Palmiter) and sometimes quite far upstream^{11,12}, but their developmental role is equivocal. Other possible developmental controls include tissue-specific differential splicing of primary transcripts as may occur in the *Drosophila bithorax* system (M. Goldsmith-Clermont, Stanford University), tissue- and stage-specific differential gene amplification as evidenced in the *Drosophila chorion* system (F. Kafatos, Harvard University) and tissue-specific differences in protein kinase levels (R. Erikson, Harvard University). So far, cascades of sigma

factors seem limited to prokaryotic and viral developmental systems (J. Pero, Harvard University).

Importantly, in a number of cases control is not simply an 'on-off' affair, but rather quantitative changes may be critical. For example, P. Cherbas (Harvard University) reported the early protein level response to ecdysone in *Drosophila* K_c cells involves an increase in the amounts of three proteins (EIP 28, EIP 29 and EIP 40) already present before induction. The main effect of Rous sarcoma virus *src* gene may be to increase the level of cellular protein kinase by 20-fold in order to cause a gross change in cellular morphology (Erikson). The pattern of gene expression can also be affected by the changing location of transposable genetic elements, as documented in maize (B. Burr, Brookhaven National Laboratory)¹³, *Drosophila* (Ruben) and yeast (I. Herskowitz, University of California, San Francisco)¹⁴. It remains to be seen what part such genomic instabilities routinely play in

developmental processes outside the vertebrate immune system.

Appropriately, Kafatos cautioned that developmental biologists should not assume that the way things are controlled in one system will be the way they are controlled in another. For example, compare two insects: the silk moth and *Drosophila*. The former constructs its eggshell using germ-line repeated gene pairs while the latter employs differential gene amplification of single-copy genes. Yet, perhaps one should not be too surprised by the difference since the lepidoptera and diptera diverged evolutionarily almost 300 million years ago, about the same time that human and reptilian lines split.

The conference ended with an appealing model from H. Weintraub (Hutchinson Cancer Research Center) concerning activation of chromatin structure¹⁵. He pointed out that concomitant with the activation of globin genes during chick erythropoiesis, DNase-hypersensitive regions appear at the 5' ends of the genes due to the formation of single-stranded stem-loop structures. Once induced these hypersensitive sites remain even after removal of the inducer and even through DNA replication. Interestingly, these sites can be induced by salt shock. Changing the salt concentration drastically alters the places where S₁ nuclease will cut. Hence, Weintraub speculates, if the early embryo contains a salt gradient perhaps this could activate different chromatin regions in cells occupying different positions within the gradient. In short, Weismann's determinants could be differential salt concentrations! □

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100 years ago

ELECTRIC NAVIGATION

THE idea of propelling a boat through water by the motive power of electricity is no new one. The invention of the electromagnet showed the power of an electric current to produce a mechanical force. It was no very difficult matter, therefore, for the electricians of fifty years ago to utilise the force of the electromagnet to drive small electromagnetic engines; and from the small beginnings of Dal Negro, Henry, Ritchie, and Page, grew up a group of electric motors which only awaited a cheap production of electric currents to become valuable labour-saving appliances. Nor was it a very long stride to foresee that if a sufficiently powerful battery could be accommodated on board a boat, it might be possible to propel a vessel with electromagnetic engines drawing their supply of currents from the batteries. This suggestion — one of the earliest, indeed, of the many applications of the electromagnet — was made by Prof. Jacobi of St. Petersburg, who, in

1838, constructed an electric boat. This machine was worked at first by a Daniell's battery of 320 couples, containing plates of zinc and copper, 36 square inches each, and excited by a charge of sulphuric acid and sulphate of copper. The speed attained with this battery did not reach so much as 1 1/4 miles per hour. But in the following year, 1839, the improvement was made of substituting 64 Grove's cells, in each of which the platinum plates were 36 square inches in area. The boat, which was about 28 feet long, 7 1/2 broad, and not quite 3 feet in depth, was propelled, with a convoy of fourteen persons, along the River Neva, at a speed of 2 1/4 (English) miles per hour.

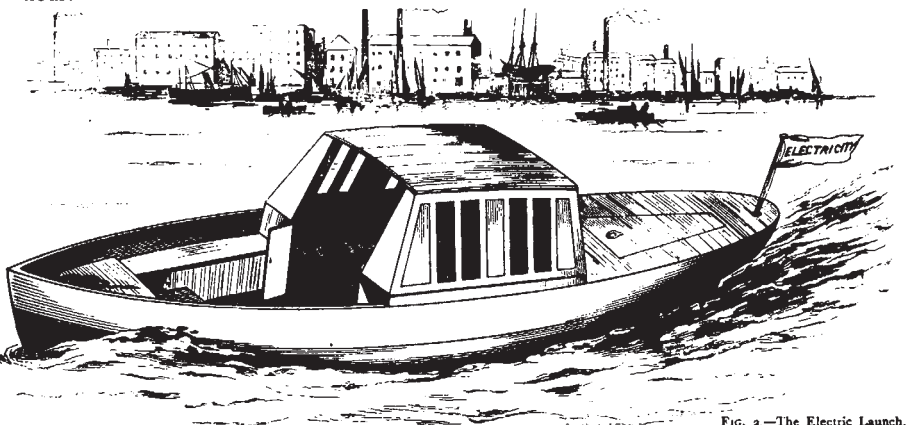


FIG. 2—The Electric Launch.

The electric launch *Electricity*, which made its trial trip on Thursday, September 28, 1882, on the waters of the Thames, is certainly a great advance upon that which had been previously attained. This boat, is of iron, and is a trifle less in length than the wooden boat which Jacobi propelled. She will carry twelve persons, though at the trial trip but four were on board. After a few minutes' run down the river and a trial of the powers of the boat, to go forward, slacken, or go astern at will, her head was turned Citywards, and she sped — I cannot say steamed — along the southern shore, running about eight knots an hour against the tide.

From *Nature* 26, 554, 5 October 1882.

Solar-terrestrial influences on weather and climate

from John Gregory

No longer is it appropriate to ask, as did one book reviewer, whether the subject of links between the Sun and weather and climate is a fit one for scientific study. The topic has now become respectable and, in the next quarter-century, will take its place among new and revised studies of solar-terrestrial relationships. These are the overall impressions gained at the recent Second International Symposium on Solar-Terrestrial Influences on Weather and Climate*. There are three main reasons for these developments; first, some previous evidence for solar-climate relations has withstood continued scrutiny; second, the variability of solar output is at last being measured; and third, new physical effects linking the Earth and Sun are being observed in the terrestrial system.

At the first symposium in 1979, Mitchell (NOAA), Stockton and Meko (University of Arizona) had used tree ring data to show that the incidence of droughts on the Great Plains west of the Mississippi is linked to the Hale (22 yr) solar cycle¹. The result has been generally approved^{2,3} and at the conference the original authors incorporated additional data in a new analysis and the 22 yr period was confirmed; though as earlier reported, it is not consistently present in all regions. Evidence for an 18–19 yr period was found, as noted by Currie⁴, in the interval 1850–1962. The phase linkage, which places drought maxima about 2 yr after Hale minima, was also confirmed, but at higher significance from 1700 to 1850 than at other epochs. The authors concluded that the variance of drought area indices may follow both the 18.6 yr lunar cycle and the 22 yr solar cycle, but that the latter is the predominant periodic component.

The best historical evidence for the short-term cyclic nature of climate continues to be the Precambrian distal varves (annual deposits of sediment) found in the Elatina formation of South Australia⁵. G. Williams, noting similarities between the 700 Myr rock and modern records, ventured a prediction of a ~300 yr cycle of solar activity, into the trough of which the Sun is now entering (see the table).

For more recent times, cores drilled in compacted snow at the South Pole and Vostok show evidence of 11 and 22 yr periodicity over about 1,000 yr. G. Dreschhoff, E. Zeller and B. Parker (University of Kansas) examined nitrate

concentrations in the cores from the Pole, and incidentally found an unambiguous decrease coincident with the Maunder minimum.

Comparison of periods of solar activity (years)		
Elatina formation	Tree rings	Solar records
11	—	11
22	22	22
90	—	90
145	142–157	—
290	270–300	—

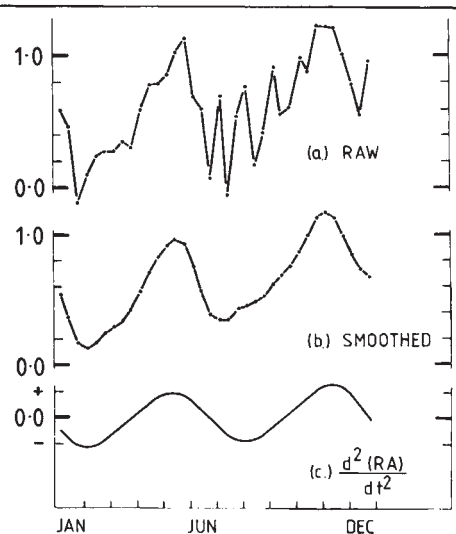
What processes might link solar activity and weather-climate? The opening speaker, J. Eddy (NCAR), asked for 'strong hints' as to mechanism and three speakers can be selected as providing answers.

D. Page (European Space Agency) analysed magnetic indices interpreted in terms of torques exerted by the interplanetary magnetic field on the magnetosphere. By subtracting magnetic indices for northern and southern hemispheres ($A_n - A_s$), and ordering them according to the polarity of the solar field, positive (away) and negative (towards), temporal variations of the quantity ($A_n - A_s$) positive – ($A_n - A_s$) negative were studied. The figure shows a basic result, which is well substantiated in subsets of data. There is an evident connection with the acceleration of the Sun in Right Ascension. To link angular accelerations back to torques, magnetospheric interaction with the interplanetary field is invoked. Temporal variations are interpreted as indicating that the torque on the magnetosphere is maximum when the north magnetic pole is closest to the Sun. Effects in the terrestrial system include characteristic differences in the recovery of magnetic storms, the phase

of the semi-diurnal atmospheric pressure tide at near-equatorial stations, which shifts about 15 minutes, and also shifts when magnetic sector boundaries are crossed, changes in average daily pressure differences between stations north and south of the equator by 1 mbar with change of polarity of the interplanetary field (the range of daily mean pressure at these locations being 2.5 and 9 mbar respectively) and the temporal occurrence of earthquakes. The build-up of torque to a maximum at about one day before a magnetic storm is also inferred from the behaviour of the D_{ST} index. A new mechanism is proposed for the origin of the semi-diurnal atmospheric tide, which would become a dynamical, not a thermal, response. No detailed mechanism which transmits torque on the magnetosphere to the neutral atmosphere and solid earth was proposed. Page points out that his interpretation requires that orbital energies be involved, and these are some 4×10^3 larger than rotational energy for a mass at the equator, in comparison with which solar radiation is much less significant. His interpretation may lead to considerable discussion as to the physics involved, but this should not be allowed to obscure the importance of the new terrestrial responses which he has established.

The second paper relates to a more familiar difficulty, the momentum balance of the stratosphere and mesosphere, which is coming closer to solution⁶. J. Gregory, A. Manson and C. Meek (University of Saskatchewan) showed, from wind measurements at 60–110 km altitude, that mean winter zonal speeds at 52°N have increased from solar minimum to maximum by a factor of at least two, the increase being largest a little above the mesopause (85 km). Little-known measurements at 95 km altitude, taken in the GDR and USSR for eighteen years⁷, support the interpretation of the Canadian data as an 11 yr cycle. In all the records, the increase in speeds is greater in winter than summer, by a factor of at least two to four in the mesosphere. To account for this,

The quantity $(A_n - A_s)$ post – ($A_n - A_s$) neg plotted at 10-day intervals averaged over 1959–74. (a) gives the single 10-day values and (b) is obtained from (a) by taking 3-point moving averages. Curve (c) is the acceleration of the Sun in Right Ascension.



*The symposium, organized by B.M. McCormac and W.O. Roberts, was held at Boulder, Colorado, on 2–6 August 1982.

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Gregory proposed that increased heating, due to enhanced UV radiation around solar maximum, is most effective in the summer tropics and hemisphere. The meridional flow from summer to winter hemispheres is enhanced, and the solar modulation is thus transferred via Coriolis torque into the winter circulation, itself inherently variable. Other work^{8,9} shows that this effect is detectable in the winter stratosphere and troposphere.

The third paper involves long-wave radiation. G. Reid and K. Gage (NOAA) have extended their work¹⁰ on the annual variation of the height of the tropical tropopause to model an 11 yr variation, with change in solar 'constant' as a key factor. They showed that there is considerable sensitivity of that height to mean sea surface temperatures, via absolute humidity and deep cumulus convection. To establish agreement with observations, an increase in the solar 'constant' on the 11 yr basis is required. Reid reminded listeners that the decreases so far observed, of a few tenths of 1 per cent, are for shorter periods, and that the

change in solar irradiance over the 11 yr cycle is not yet established.

The symposium heard substantial papers on the variations of solar input to the atmosphere, induced effects in the upper atmosphere, responses of climate models to solar perturbations and also many papers on short-term effects and favoured mechanisms. These will appear in the proceedings. Special mention may be made of a survey by M. Nicolet (Pennsylvania State University) of photochemical responses affecting ozone: this comprehensive study will be a valuable reference source. □

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numerous kind of variable star in the Galaxy, in large part because white dwarfs themselves are so numerous (perhaps 10 per cent of all stars in the Galaxy). The variable DB white dwarfs may turn out to be the second most numerous kind of variable star in the Galaxy.

The new discovery is particularly significant because it confirms many of our ideas regarding stellar pulsation and its causes in general, and variable white dwarfs in particular. By applying pulsation theory to certain details of the observed period structures, it is possible to infer, for example, the rotation periods of the white dwarfs of interest, something that has been difficult to do by other methods. This has now been done for several variable white dwarfs. For the GD358 the method yields a rotation period of about 1.5 hours, which is quite representative of periods for white dwarfs whose rotations have been studied.

By applying pulsation theory to other aspects of variable white dwarfs, it is possible to study their internal structure in much the same way geophysicists use seismic waves to study the interior of the Earth. For example, that only certain periods are present in the variable white dwarfs strongly suggests that the outer regions have a layered structure — the thin hydrogen and/or helium layers can 'trap' certain pulsation modes⁵. The layered structure had previously been inferred on the basis of other elements of stellar evolution theory⁶. Since nearly all DA white dwarfs are expected to go through a variable phase sometime in their evolution, the layered structure is probably found in white dwarfs in general.

Similarly, it should be possible in the future to use the pulsations of GD358 to infer something about the interiors of the DB white dwarfs. It is interesting that the 'driving agent' for the pulsations of the DB white dwarfs is second helium ionization, as is thought to be mainly responsible for pulsations in the classical Cepheids. On the other hand, the same theory that led to the prediction that GD358 should be variable has identified the main physical cause of the pulsations of the ZZ Ceti stars as hydrogen ionization^{1-3,7,8}. This is the same physical agent that is believed to be mainly (or at least largely) responsible for the pulsations of the Mira variables⁹, among the largest stars known. □

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A new type of pulsating star

from John P. Cox

A NEW type of pulsating star has been found by astronomers at the McDonald Observatory in Texas. The newly discovered variable star, known as GD358, is unique in that it is the first variable star to have been predicted as variable before its discovery^{1,2}.

The discovery, made by D.E. Winget, E.L. Robinson, R.E. Nather and G. Fontaine, was announced at the recent conference on 'Pulsations in Classical and Cataclysmic Variable Stars' held in Boulder, Colorado³. A more detailed account will be published soon in the *Astrophysical Journal*.

The new variable star is a kind of white dwarf — a DB white dwarf — whose outermost layers are composed of nearly pure helium. The surface temperature of GD358 is probably close to 30,000K. Several periods have been observed in GD358, ranging from a little more than 2 minutes up to about 16 minutes.

White dwarfs are extremely dense stars, with a mass equal to about that of the Sun and a radius comparable with the Earth's. Hence their densities are quite large — some 10⁶ times the density of water — and their surface gravities approximately 10⁵ times that of the Earth. White dwarfs are thought to represent the end stages in the evolution of at least certain kinds of stars.

Consequently, the interiors of the white dwarfs are likely to be devoid of hydrogen and, in most cases, of helium also. The hydrogen and helium often observed on the surfaces of white dwarfs is thought to be present only in thin 'films' formed when these lighter elements float to the surface under the influence of the strong gravitational field. In contrast, in most ordinary stars, the hydrogen and helium is believed to be distributed fairly uniformly throughout the interior.

The DB white dwarfs comprise only a small fraction, some 10 per cent, of all white dwarfs. Most, perhaps 80 per cent, have predominantly hydrogen in their outermost layers, and are called DA white dwarfs.

In the past decade or so, a number of variable DA white dwarfs have been found⁴. All have surface temperatures in the range 10,000–12,000K and are called 'ZZ Ceti' stars. Their periods range from about 2 to 20 minutes. The pulsations are thought to be nonradial, with different parts of the stellar surface moving out of phase with each other. The variable DA white dwarfs are thought to be ordinary DA white dwarfs which have slowly evolved into a condition where they pulsate for a time, perhaps a few million years — like temporary 'sickness' — and then become ordinary, nonvariable DA white dwarfs again.

Although less than 20 ZZ Ceti stars are known, they are probably by far the most

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THE AUTOMATED LABORATORY

A load off the mind

AUTOMATION in the laboratory is like automation anywhere, but more so. Immediately after the Second World War, it seemed in all kinds of laboratories to be sensible that the repetitive measurement of the same properties of similar samples should be made by using the techniques developed by mechanical engineers for assembling bits and pieces of motor cars into standard structures — plastic bottles containing predetermined amounts of solutions doped with known radioactive isotopes would in this spirit be carried within the range of suitable detectors, scintillation counters perhaps. Other repetitive laboratory jobs — the chemical analysis for specified elements of broadly similar specimens of material, for example — were made more or less automatic once there had been developed reliable devices for measuring small amounts of chemical reagents (usually in liquid form). Economically, such devices were easily justifiable; better that routine measurements and assays should be carried out by standardized procedures undisturbed by the personal equations of the experimenters, while the cost of employing laboratory assistants to carry out tasks that could be delegated to machines was often higher than that of the interest paid on large sums of money borrowed from some bank. Production-line automation in this style remains as productive of improved efficiency as at any time in the past three decades. Automatic amino acid analysers, or machines that will assemble some predetermined nucleotide sequence from its component nucleotides, have become essential components of many laboratories' equipment.

The notes in the pages that follow show, however, that the ambitions of those who would make laboratories automatic have now transcended those fashionable in earlier decades. At the bench, or at the control panel of some large telescope, people are now rightly concerned to ask whether the arrival of microprocessors provides an opportunity for delegating to machines tasks that can in principle be carried out only by people, but which are in practice likely to be performed only hesitantly, even inefficiently. Of all automatic laboratory instruments, the machine-driven telescope (dating from the early nineteenth century) is the prototype: even clockwork machinery is usually more dependable than a human being at following the rotation of the Earth on its axis.

Traditionally, astronomers have supposed that only human beings (people like themselves) can decide for how long a telescope should be locked onto some celestial object of particular interest before the equipment they are using can be pointed at something else. (Given the logarithmic response of the photographic emulsion to integrated light intensity, mistakes have traditionally been commonplace.) Fortunately, the high cost and, more to the point, the competition for access to machines such as the Einstein X-ray observatory have reminded astronomers of the need to work out in advance of the collection of radiation from a nominated celestial object the amount of radiation (the number of quanta) that will yield a significant result. One obvious snag, of course, is that the significance of a data-set thus collected automatically may depend critically on the quality of the data, so that it will not be possible to specify in advance how many quanta should be collected but only to work out as the data accumulate when an observation should be stopped. That is a problem that those who design experiments in high-energy physics have lived with for several years. What they have found is that the automated laboratory brings severe and

unusual disciplines. The hypothesis to be tested must be clearly specified, and there must be explicit criteria for deciding what constitutes a significant test. Not unnaturally, in fields where the choice between manual and automatic experimentation is not overwhelmingly determined by the decrease of cost that automation brings, people resent the need to be this clear in advance.

These uncharted fields happen also to be those in which there is at present most interest, among manufacturers and their customers, in the automation of laboratory equipment. Two important influences have helped to bring about this state of affairs. First, the proportions of working scientists now reasonably skilled at writing computer programs has surreptitiously increased so that the notion that an experimental program might with advantage be delegated to a machine is more likely to come up than not. Second, the microprocessor has become ubiquitous and at the same time cheap. It is therefore natural enough that there should be a rash of laboratories concerned with traditional fields of research, muscle physiology or old-fashioned biochemical investigations, in which ingenious people have found it worthwhile (or at least fun) to latch together a microprocessor (perhaps in the form of a 'home' computer) and an experimental rig in such a way as to avoid the need for telling when one 'run' is at an end, and that it is time for another to begin. It is more of a surprise that equipment manufacturers rightly alert to the opportunities for adding to their catalogues equipment incorporating microprocessors should so often have found it necessary or at least expedient to quote substantially increased prices for automatic equipment compared with the traditional variety. Although users appreciate the need to amortize the cost of new developments, and even appreciate that their own increased efficiency is worth paying for, there is also a danger that manufacturers will rob themselves of valuable opportunities, and deprive their customers of important benefits, if they persist in seeking for themselves the whole of the value that can be added by automation.

In reality, the developments of the past few decades in the automation of experimental work are probably no more than a modest and even grudging concession to the practical importance of computing machinery. So much, it is hoped, will be apparent from Professor Donald Michie's article on computer chess (see page 391). This game is properly regarded as a kind of simulation of the problem of understanding how the natural world is put together. There is a qualitative correspondence between the search for winning strategies and that for successful hypotheses about the origins of natural phenomena. Computationally, chess is for the time being beyond the reach of even very large computers, implying that the understanding of natural phenomena is even less likely to be turned over to machines. But the past decade has seen such remarkable improvements in the capacity of machines to play the mechanical game of chess respectably, and the way ahead so clearly points to the utility of abstract definitions of useful strategies, that it is not unreasonable to hope that the practice of science may in due course be rid of the tedium by which generations of students (and even their teachers) have been oppressed. The drift towards the automation of the laboratory may be sustained by economic calculations turning on the salaries of technicians, but the long-term objective must be to free the imagination, not to cramp it.

The manufacturers' approach

THE means to automate laboratory instruments have been available for some years. Laboratories with large mainframe computers or minicomputers have been able to use them to automate certain aspects of instrument control and data processing. But developing such systems around large computers is costly. Automation has only really been feasible for many applications in the few years since the development and availability of cheap microprocessors. Manufacturers of scientific equipment nowadays are always keen to point out that their newly-developed instruments, even the cheapest, are in some way microprocessor controlled.

Researchers and technicians on the whole have welcomed automation, although some notable problems do exist (see opposite). Automated instruments are often easier and quicker to use than old methods and in many cases they make feasible experiments and measurements that would otherwise have taken too long to be seriously contemplated.

Control or data handling

Microprocessors or complete computer systems can be used in scientific instruments for two different but related functions — instrument control and data processing. The way in which the computer unit is incorporated into the instrument is crucially important for it determines the degree of control the user has over what the instrument will do. In general, microprocessor units pre-programmed in read-only memory cannot be easily re-programmed. Thus, the user has to accept the instrument functions that the manufacturer has decided he or she will want to do. Many users are only too willing for the microprocessor to take over the tedium of setting

up and continually adjusting instruments. Others find the manufacturers' programs too limited for their purposes.

Manufacturers have attempted to overcome the problem of satisfying different types of user in different ways. They are still searching for solutions. But in general, cheaper instruments include a microprocessor unit, pre-programmed in unerasable read only memory, that is responsible for both instrument control and, where applicable, data processing.

Pre- and re-programming

Moderately-priced instruments, worth up to a few tens of thousands of pounds, may include a pre-programmed microprocessor unit for instrument control and a standard interface for feeding data output from the instrument into a computer for data processing. Although the microprocessor unit may not be designed to be re-programmed, manufacturers can often program it to perform a wide range of functions including the sophisticated control of several parameters by dynamic feedback. Theoretically at least, users can connect their instrument to any computer for data analysis but in practice the choice of computer is often limited to those for which the manufacturer has written software for data analysis. Writing software from scratch for a different computer operating system is usually far too expensive — almost certainly more expensive than modifying the manufacturers' software considerably.

Manufacturers normally offer users the capability to re-program all aspects of instrument control and data processing only for costly instruments at the top end of their ranges. The reason is not too difficult to appreciate. A microprocessor unit pro-

duced as pre-programmed hardware is cheap and can be made to satisfy the bulk of users who may be technicians working in industrial or testing laboratories. Offering the researcher access to all aspects of instrument control may involve providing an interface for a separate microcomputer to over-ride programs written into the microprocessor unit. That is costly.

One example of a medium- to high-priced instrument is the X-ray spectrometer developed by the Division of Science and Industry at Philips in Eindhoven, the Netherlands. A pre-programmed microprocessor unit controls the instrument and a standard interface is provided for outputting data to a stand alone computer. The user selects the control program by answering a series of questions that the instrument displays on a screen. Safeguards are included so that user cannot enter a potentially dangerous command. Once a program has been selected it can be stored for recall later, perhaps by a completely inexperienced operator.

Until recently, Philips has always regarded its ultraviolet and atomic absorption spectrophotometers as falling into the cheaper category of instrument. Hence it has provided them with only a single microprocessor pre-programmed for instrument control and data analysis. Now, however, the scope of the instruments can be extended by adding a programmable calculator and automatic sample changer.

Investment in software

Before the advent of the cheap microprocessor, an instrument manufacturers' investment in developing automated systems would have been mainly on hardware. Now, it is not surprising that most effort goes into software development. The instruments that have perhaps been most successfully automated are those concerned with analytical chemistry. A large market for analytical equipment that is cheap and easy to operate exists not only in routine testing laboratories but also in research. Chromatography, mass spectrometry and liquid scintillation, for example, have many uses.

A look at the development of liquid scintillation counters by Philips illustrates how microprocessor development has influenced the way in which the company regards future instrument development. Not so many years ago, the instrument's computer control system was a series of boxes piled on top of each other, the system taking more space than the liquid scintillation counter itself. Now the microcomputer unit is housed in a small box under the instrument.

Although the basic instrument has changed little in outward appearance, the miniaturization of the control system has meant that more features can be programmed into it. Today's instrument contains a pre-programmed unit containing 16 programs corresponding to the most commonly used isotope labels in

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

samples. The user can load several trays, each containing ten samples, into the instrument. Each tray carries instructions on the program by which its samples are to be analysed. The instrument can be connected to a microcomputer for data analysis if desired. Philips envisages further development in this range of instruments occurring when a new generation of microprocessor chips is available. The instrument will probably look much the same but more microprocessor power should make it faster or give it extended capabilities.

Mechanical limitations

But more advanced automation does not depend simply on the advent of the 16 bit or even 64 bit microprocessor. For some applications, advances in mechanical engineering seem to be lacking behind computer control. High performance liquid chromatography, for example, has reached the stage where further improvement will only be made if mass spectrometers can be used as detectors. And that involves using microbore columns with very slow flow rates. The development of microbore columns, which must be packed with suitable phases, now limits further improvements in the sensitivity of HPLC.

The bane of many laboratories especially those involved in routine testing, is the preparation of large numbers of samples. Unfortunately, however, few instrument manufacturers have either had the expertise or considered it economically worthwhile to develop automatic sample preparation. Hence, for example, the user of Philips liquid scintillation counter may be able to load up the machine to analyse several hundred samples in one batch but someone probably still has to put each sample into its bottle in the first place. Sample preparation could ultimately be the automation log-jam.

Judy Redfearn

When to go it alone

THE laboratory microcomputer and the automation of laboratory instruments have, in general, been welcomed by potential users — although some will always prefer to exercise their hard-won skills in setting up difficult experiments by manually adjusting knobs, carefully measuring angles and the like. The general enthusiasm for automation, however, does not always extend to the equipment on offer. The more a laboratory or organization is involved in fundamental research, the less likely it seems to be satisfied with the automated laboratory instruments that can be bought off the shelf. Such organizations are likely to devote considerable resources to custom-built instruments and computer systems.

Instrument manufacturers, not surprisingly, have developed equipment that will do the job of the majority of users as cheaply as possible. Thus, much analytical equipment is geared, for example, towards the needs of the testing laboratory in industry or medicine. Researchers may find that the commercially-available equipment of the type they are looking for does not have the flexibility of operation they desire. Only in the case of the most costly instruments may it be in the manufacturer's commercial interest to devote resources to altering the instrument to meet the customer's specific needs. In the majority of cases the task, then, is up to the research customer.

Assessing the economics of do-it-yourself automation is complex and, it seems, not often undertaken. Off-the-shelf equipment is undoubtedly much cheaper than custom-built. But to what extent should a laboratory strive to make do with off-the-shelf at the risk of compromising a

piece of research? Some university departments seem to think never — instrument development is often seen as an integral part of a piece of research and as much is said in grant applications. Industrial research laboratories, with their more mission-oriented approach to research, however, may be more willing to use what equipment is available and to try to adapt it if necessary.

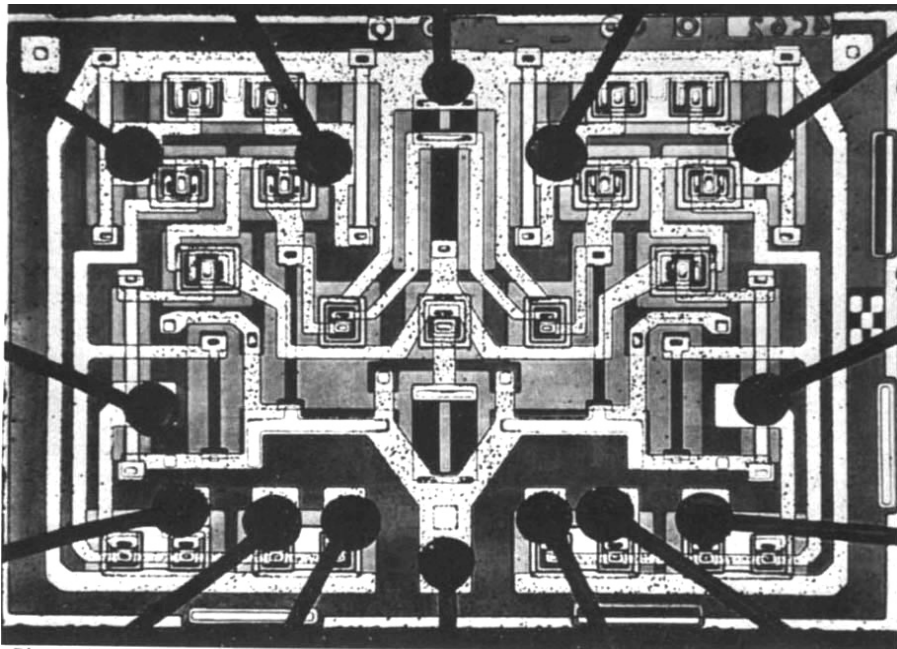
The problem is that the relatively cheap ranges of instrument, those that are most commonly used, often contain pre-programmed read only memories that cannot be re-programmed. The ideal, according to Dr Nick Goddard from the chemistry department at Imperial College in the University of London, would be for manufacturers to incorporate an interface into their instruments allowing users to feed their own programs into the built-in microprocessor. As that rarely happens with cheap instruments, users resort to building their own using standard subsystems and modules wherever possible. Occasionally, a research team may embark on designing a microprocessor system from scratch (see p.406), but generally it makes far more sense to control a custom-designed instrument using a standard microcomputer.

Custom-building

Two examples of custom-built automated instruments at the Imperial College chemistry department illustrate very different reasons for designing instruments. In one, the team is trying to devise a relatively cheap method of measuring very small concentrations of copper in solution by relying on the fact that copper will plate out on an electrode. The electroconductivity equipment used is controlled by a program written for a Research Machines microcomputer. Various programs for data analysis have also been written. The idea is to measure small concentrations of heavy metals as accurately as and more cheaply than spectroscopy.

The other development is driven by the need to measure transient effects in an electrode, which no commercially-available spectrometer can apparently do at present. The team is combining electrochemistry and spectroscopy in a technique to record a spectrum in a matter of microseconds.

An industrial research laboratory's problems with automation may be very different from those of a university, chiefly because research is mission-oriented and often on a large scale. Sample preparation and handling, for example, can be limiting. The Wellcome Research Laboratories at Beckenham in Kent, part of the Wellcome Foundation, have tackled custom-designed automation by establishing a



Photomicrograph of a double-logic gate silicon integrated circuit

special automation department of 50 or so people, nominally linked to the physical chemistry laboratory, but now acting as consultants to other laboratories on the Wellcome site. Eleven of the team are specialists in electronics or mechanical engineering, the rest being physical chemists.

Among the systems developed by the team and now operating is a method of controlling and measuring fluorescence polarization spectra, using an Apple microcomputer interfaced to the fluorescence instrument and a DEC PDP 11 34 minicomputer. The system basically speeds up the analysis of fluorescence polarization data. The Apple microcomputer logs data and controls the basic functions of the instrument. Data can be analysed slowly on the Apple or more rapidly on the PDP 11 34.

In another development, the team claims to have completely automated a Fourier transform nuclear magnetic resonance spectrometer made by Bruker. The mechanical engineering division devised an automatic sample changer made from non-ferromagnetic material and the electronics division wrote programs to control the sample changer and certain of the spectrometer functions with an Apple. Final data are analysed with software written for a PDP-11/15. The chief advantage of the modification is that an otherwise standard Bruker instrument now operates untended outside working hours.

Not all the Wellcome group's automation projects, however, have involved the application of computers or microprocessors in novel ways to established instruments. The skills of the mechanical engineering department have in some cases been sufficient to automate a process fully usually by automating sample handling or preparation. One notable example, still under development, is an automatic technique for preparing a sample for infrared spectroscopy. The key to the technique involves grinding the sample in a small pot with a vane that works like a reverse Archimedean screw and then automatically compressing it into a thin flat disk.

The automation group, according to Dr A. Everett, head of the department of physical chemistry, has had such success with some of its projects that instrument manufacturers, not often keen to modify instruments for one particular customer's needs, have been keen to help. The laboratories' policy is to encourage manufacturers' assistance in developing a technique rapidly — manufacturers, of course, benefit from the improvements they can subsequently make to their instrument. Dr Everett is convinced that his laboratory has developed sufficient automated techniques to set up in the instrument business on its own. But the practical difficulties involved have persuaded Wellcome to concentrate instead on forming comfortable symbiotic relationships with existing instrument makers.

Judy Redfearn

Astronomy — by proxy

ONE of the perks that makes it worthwhile being an astronomer in the United Kingdom — the chance to visit Hawaii — may be harder to come by as a result of a new 7,000-mile computer link-up. On 6 September an astronomer sitting at a computer terminal at the Royal Observatory in Edinburgh used the UK Infrared Telescope at Mauna Kea in Hawaii to take an infrared photometric measurement and a spectrophotometric scan of the star HR 8824.

As well as saving on travel costs between Britain and Hawaii (the initial 3-hour test cost only £80), the new system will make the operation of the telescope much more flexible, with night-time observations in Hawaii being carried out during normal working hours in Edinburgh.

The route between Edinburgh and

Hawaii is complex and instructions take around 6 seconds to make the trip. Messages pass from the observatory's computer console via the Edinburgh Regional Computer Centre to the Science and Engineering Research Council's Rutherford Appleton Laboratory in Oxfordshire.

British Telecom then takes the signal to the International Packet Switching Service and via transatlantic satellite link to the American packet switching network Telenet. The message emerges from the Telenet network at Honolulu in Hawaii and then goes by telephone line to the Mauna Kea laboratory's remote control room at the foot of the mountains. Only then does the message travel the final 14,000 feet to the observatory in the clear seeing conditions on the mountain peak.



Telescope installations on Mauna Kea — the 150-inch UK Infrared Telescope is on the right

Microprocessors — what are they?

THE basic elements of a microprocessor system are fourfold: the central processor unit (CPU), the program control unit (PCU), the memory and the input/output devices (or 'peripherals').

The input/output devices usually encountered with mainframe and minicomputers are visual display units, keyboard writers and printer. All of these devices can be connected to microprocessors, but the advantage and cheapness of microprocessors for the experimenter are perhaps more significant with less sophisticated peripherals. For instance, the pressure of a gas, normally relayed electrically to a chart-recorder or some other analog device, can be fed directly via an analog-digital convertor

into the input of a microprocessor. A trivial task for the microprocessor might be to adjust the frequency of measurement according to the rate of change in pressure. The output of the microprocessor might be connected to a gas heater, so that the 'boundary condition' of the experiment could be altered depending on the results obtained.

This interactive system exemplifies the flexibility and usefulness of microprocessors: only a few chips are needed to handle such a system — memory chips and processor chips.

There are two principal types of memory chip: random access memories (RAMs) and read only memories (ROMs). The former are used for storage and recall of

data during the running of a program, while the latter are pre-programmed before incorporation into the microprocessor system. The ROMs are programmable in several ways. Mask programmable ROMs are programmed during construction by the manufacturer and, being economic only in large production runs, are unlikely to be of use to the individual experimenter seeking a tailor-made system.

The field-programmable and erasable-programmable ROMs (EPROMs) can be programmed by the user although usually this involves specially designed

Abbreviations

CPU	Central processor unit
PCU	Central control unit
RAM	Random access memory
ROM	Read only memory
EPROM	Erasable-programmable ROM

programming devices. Once programmed, however, the chip remains so, whether or not it is plugged into the system, until erased by a particular type of voltage pulse, or by shining ultraviolet light through a window above the integrated circuit in the case of some EPROMs. Of course, once the system is developed and working the microprocessor program is fixed, so that such time-consuming procedures are limited only to construction and development.

Computer games

RAMs incorporate a two-dimensional matrix of semiconductor on-off switches, each of which acts as a unit (1 or 0) of digital memory and each of which is accessible by combining their 'x' and 'y' coordinates into a digital word. As miniaturization proceeds so the size of matrix that can be incorporated into a single chip increases. This is one of the important battlegrounds of today's micro-computing manufacturers. [As the size of memory increases, so does the number of 'bits' (binary digits) required to access it. For instance, a 256 bit memory requires 8 bits to access its individual locations, that is, physically speaking, 8 wires connecting it to the central control processor ($256 = 2^8$).] Currently RAMs are available with memories of 16K (16,000 bits), 32K and 64K and the 246K RAM is just around the corner. The 1M RAM is on the horizon (Nippon Electric Company recently unveiled a 1M ROM). The CPU carries out the arithmetic instructions stored in the ROM, as coordinated by the PCU. Typical operations are ADD, SUBJECT, SHIFT (left and right), logical AND, OR etc. Such operations are not only essential for calculation but can be used with ingenuity, by manipulating or monitoring individual digits within binary numbers to produce very fast and efficient programming. As with much programming, the development of such lateral thinking is great fun and becomes an enjoyable means of procrastination.

The final component of the microprocessor system is the PCU. This undergoes a cycle of process that coordinates and instructs the other components in a sequence which is maintained by an internal clock. This 'cleaning house' activity is managed via several 'registers' inside the PCU. A typical cycle might be as follows:

(1) A program control counter, whose current value defines the memory 'address' of the next program instruction within the appropriate memory (usually a ROM), feeds its number into the memory access register of the PCU.

(2) The instruction at this address (which will be coded as a single binary number) is transferred from the memory into the 'instruction register' of the PCU.

(3) The instruction register is read and appropriate semiconductor switches in the central processor unit are either

opened or closed.

(4) The program control counter is incremented by 1.

The programming 'language' used with a microprocessor system can be a standard language (for example, FORTRAN) which is translated into the unique code of the microprocessor system via a compiler. The advantage is the relative ease and familiarity with the programming but the disadvantages are the necessity of a compiler and an inefficient (in terms of memory space and speed) use of the microprocessor. By using the direct code of the microprocessor on the other hand, the system will be more efficient but will be correspondingly more difficult to develop and 'de-bug'. Whichever route one takes, and whether or not one asks a consulting agency to do the job completely, will depend on experience, time and money available, and the complexity of the required system.

Philip Campbell

Microcomputers for all

NOT so long ago, researchers had very little option other than to communicate with a mainframe computer when they wanted to analyse data from their experiments. The experiments themselves could be controlled by computer, either by a mainframe or a minicomputer available for the use of, say, one laboratory — but only if someone was available with the necessary expertise to do the hard wiring and write the software. Now, however, cheap, powerful microcomputers are altering the balance of computing power in research organizations and universities.

Microcomputers have fallen so much in price in recent years that many researchers, even apparently in Britain's financially hard-pressed universities, can afford to buy one out of their research grants or general budgets. Groups of two or three researchers with their own shared microcomputer are commonplace and even the sole researcher with his or her own personal computer is not uncommon. Microcomputers may not be able to do everything a mainframe computer can — their reduced power, for example, means that they are far slower at detailed and lengthy calculations — but they do contain substantial computing power packaged in a very useful form. Microcomputers can be used, for example, far more easily than mainframe computers, to control laboratory experiments. They can also be converted to word processors simply by buying suitable software.

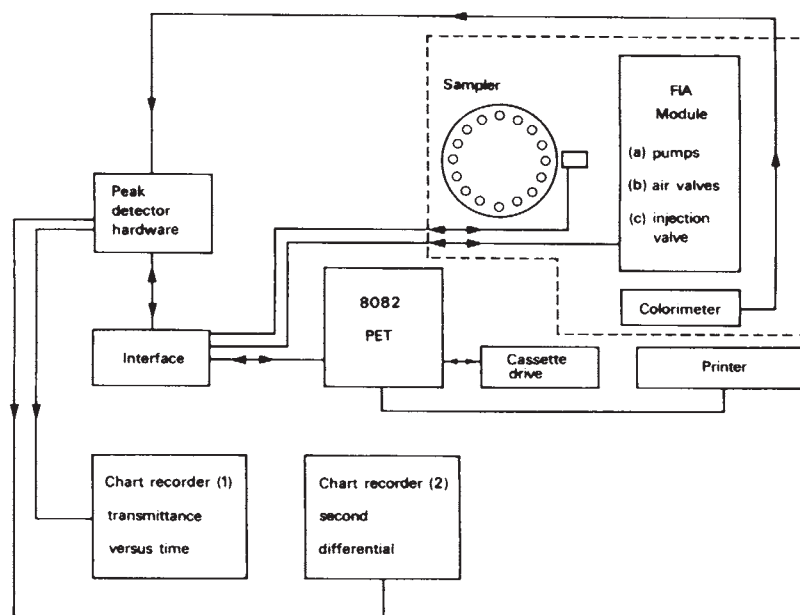
Proven software

The microcomputer's usefulness to laboratory scientists stems largely from the fact that they are proven systems for which a lot of software has been written, not only of the type needed, say, for word proces-

sing, but also for statistical analysis and even for control of experiments. Even if the specific software for a particular application is not available, writing it or adapting old software is not usually too much of a problem given that the popular ranges of microcomputer, at least, use standard and proven operating systems. Some researchers will always believe that nothing is available off the shelf that would do the job they want. They will probably design a microcomputer system from scratch, perhaps contributing to microprocessor development in the process. But for most researchers who have no special expertise in microprocessor design and whose aim is to spend as much time as possible on research in their particular discipline, adapting standard microcomputers to their singular needs is undoubtedly most cost effective.

The researcher with a microcomputer, however, may not necessarily find life plain sailing. He or she would normally rely on the services of a central computing unit to help with problems on the mainframe. Now, however, the computing power is entirely within the researcher's control and specialist computer staff may not be available to help with problems. The researcher needs to know at least the basics of computing and to understand the capabilities of his or her microcomputer.

Universities have started to run courses aimed at the researcher who would benefit from using a microcomputer but knows little about them. One such course, run by the chemistry department and computer centre at Imperial College in the University of London, has attracted support from the British Department of Industry which needed a means of training scientists in government laboratories, especially the



An example of a layout for microcomputer control of hardware

Laboratory of the Government Chemist, to make better use of microcomputers. The course, held twice a year, runs for one week and is open to anybody who can pay the £300 attendance fee. The industry department provided £60,000 to set the course up eighteen months ago under its Microcomputers Applications Programme (MAP). Most of the money was spent on setting up demonstration experiments and writing course software.

According to Edward James of Imperial College computer centre, the course has two aims: to help people who know nothing of microcomputers, even those who have difficulty understanding the manufacturer's unpacking instructions, and to make them realize the microcomputer's full capability. Another aim is to help researchers choose the right microcomputer system — the wrong system could cause chaos, says Mr James.

The course begins with an introduction to how a microcomputer stores and transfers information and how it executes programs. Participants learn how to program in BASIC. The capabilities and limitations of standard interfaces such as the IEEE-438, a favourite with manufacturers of scientific instruments, are discussed — and so too are the practicalities of converting analog signals to digital and vice versa. Participants are also introduced to programming languages other than BASIC, including machine codes.

Practical help

Nick Goddard from Imperial College's chemistry department designed a special non-standard interface for the course which allows the user to interface up to 16 of any combination of function boards into a microcomputer. He and other members of the chemistry department also devised a series of experiments showing

participants how microcomputers can be used in practice and inviting them to write their own simple programs.

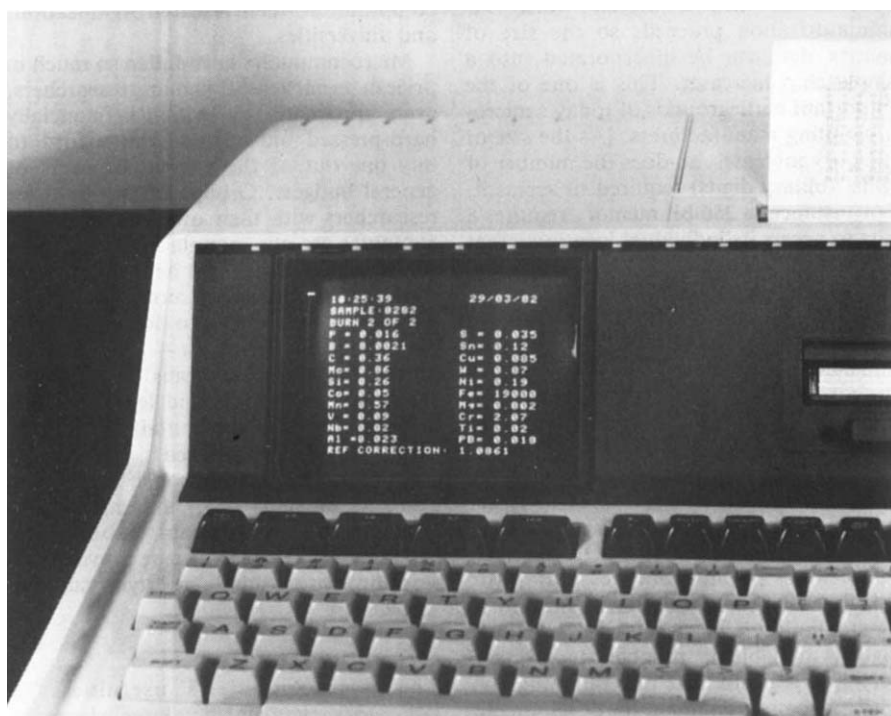
One experiment, for example, introduces course participants to computer controlled experiments by inviting them to perform acid-base titrations using standard alkali solutions delivered by a computer-controlled dispenser. Programs for calibrating the pH electrode, sampling and displaying its output at regular intervals and creating a user file containing pH results are provided. Participants are

invited to write their own programs for finding the end-points from the pH data and calculating the number of moles of acid in a sample.

The idea is that participants will return to their laboratories able to control simple experiments and analyse data with their own microcomputers. Those who do not have a "micro", but are contemplating buying one, will have a better idea of what type of system they need for their purposes and what limitations to expect. The course philosophy seems to be to recommend to those who have little computing experience systems that are well-proven with plenty of written software, even though the system capabilities may be less in theory than those of other systems. Microcomputers based on the new 16-bit processor, for example, would probably be recommended only for those who need the extra computing power and who have sufficient experience to write software from scratch. Others would be wiser to choose one of the standard 8-bit machines, for which plenty of software now exists.

One problem encountered by the Imperial College team is that of researchers who have bought instruments containing pre-programmed microprocessors only to find that the programs are not suitable for their purposes and that nothing can be done about re-programming. Another function of the course is to help researchers interface their own micros to computerized instruments where that is possible and where it is not, at least to make them aware of the pitfalls to be avoided when purchasing new equipment.

Judy Redfearn



A mission-oriented computer: A Hewlett-Packard HP-85 personal computer is incorporated in the Philips PV8020 emission spectrometer. The computer produces results in element concentrations on the built-in video screen as well as hard-copy printout.

ARTICLES

Proterozoic age and cumulate origin for granulite xenoliths, Lesotho

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Rare earth element (REE), $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ analyses on a range of granulite xenoliths from kimberlite pipes in Lesotho demonstrate that useful whole rock ages can be obtained from such lower crustal material. The characteristic positive Eu-anomalies, relatively low REE abundances and large variation in Sr contents suggest that many of the xenoliths are crystal cumulates rather than residua after partial melting. They yield an Sm–Nd whole rock age of $1,400 \pm 100$ Myr and are unrelated to the younger magmatic rocks of the Karoo.

COMPARATIVELY little is known about the age and composition of the lower crust, particularly in apparently stable continental areas. The Cretaceous kimberlites of southern Africa contain a large range of crustal material included as xenoliths, and thus offer a unique opportunity to investigate the chemical and temporal variations within a relatively large segment of continental crust. Kimberlites occur both on the Archaean craton and in the surrounding Proterozoic mobile belts, but controversy exists over the nature of the belts and the position of their boundaries within the craton beneath the Karoo cover. Kröner¹ has argued that such mobile belts are formed by reworking of pre-existing crust, but Barton *et al.*² have recently demonstrated that in both Natal and East Namaqualand a considerable amount of new crustal material was generated in these late Proterozoic events (1,300–1,100 Myr). Similarly it has been postulated that diamondiferous kimberlites tend to occur above old, cold and thick lithosphere and should therefore be confined to the cratonic areas³, and that anhydrous granulites are only found around the edges of the craton⁴ (Fig. 1). Yet in Lesotho, diamonds and granulite xenoliths are typically found in the same kimberlite pipes.

We report $^{143}\text{Nd}/^{144}\text{Nd}$, $^{87}\text{Sr}/^{86}\text{Sr}$, and trace element abundances on a suite of granulite xenoliths from Lesotho in an attempt to determine (1) whether these meta-igneous rocks represent 'liquid' compositions, cumulates or residua after partial melting; (2) the age of the lower crust in this area, and (3) any relationship with the widespread magmatic rocks of the Karoo.

The Lesotho granulite xenolith suite has been extensively analysed for both whole rock major element and mineral compositions^{4–6}. The mineralogy is predominantly clinopyroxene, garnet and plagioclase, although orthopyroxene and amphibole do occur in some samples, and accessory minerals include mica, rutile, scapolite and occasional apatite. Application of standard geothermometers and geobarometers to minerals in the samples analysed here gives temperatures and pressures in the range 500–700 °C and 5–13 kbar (ref. 4); although pressures of 15–20 kbar were also inferred from similar Lesotho granulite xenoliths by Jackson and Harte⁶, who suggested that some may therefore have been derived from depths below that of the present day Moho (~37 km)⁷. Major element analyses of most xenoliths listed in Tables 1 and 2 are similar to transitional olivine basalt with minor nepheline or hypersthene in the norm: the exception is LT 2 which is a more leukocratic granulite of intermediate composition, with significant modal and normative quartz⁴. From a consideration of the densities of the xenoliths and crustal seismic velocities beneath southern Africa, Griffin *et al.*⁴ suggested that the Lesotho lower crust comprises 50–70% basic garnet granulite and 50–30% intermediate granulite.

Trace element analyses of the xenoliths were undertaken by Griffin *et al.*⁴, and the REE abundances of five samples were reported previously⁵. In both cases it was shown that the trace elements are much more variable than the major elements, and that this variation was not caused by contamination from the trace element enriched host kimberlite. Although the granulite xenoliths were interpreted as a coherent suite of meta-igneous rocks it remained unclear whether the geochemical variations were due primarily to partial melting or fractional crystallization. In addition, the more immobile trace elements offered no unambiguous indication of the petrogenetic affinities of the xenoliths; for example, on a Ti–Zr–P discriminant diagram three of the xenoliths plot as alkali basalts, 10 as tholeiites, and the remainder fall outside any defined field⁴.

Rare earth elements

The results of the REE analyses are listed in Table 1 and illustrated in Fig. 2. The basic granulites appear to fall into two groups on the basis of their total REE abundance. Those in one group have Sm contents of 25–35×chondrite and no significant Eu anomalies, whereas the remainder have much lower REE contents (Sm = 3–11×chondrite) and typically exhibit marked positive Eu anomalies. Both groups include LREE enriched and LREE depleted samples. Interestingly, however, three rocks in the second group combine low middle REE concentrations with a slightly curious enrichment in the

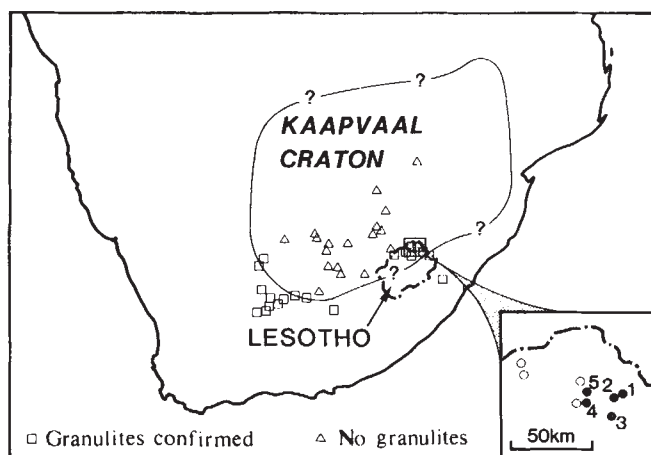


Fig. 1 Distribution of granulite xenoliths in kimberlite pipes in relation to the boundary of the Kaapvaal Craton (after ref. 4). Inset shows the xenolith localities in North Lesotho; 1, Letseng-la-Terae; 2, Mothae; 3, Matsoku; 4, Pipe 200; 5, Lihobong.

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light rare earths (samples L 13, PHN 2533 and PHN 1670, Fig. 2) and at least two of them are believed on isotopic grounds to have been affected by interaction with the host kimberlite (see Fig. 4, and discussion). Thus the LREE enrichment in such samples may be due to contamination en route to the surface. The felsic granulite LT2 is again different from the other samples in that it possesses a more fractionated REE pattern (higher La/Yb ratio), and a slight positive Eu anomaly similar to many felsic granulites from regional metamorphic terrains⁸.

Previous discussions have accepted that the granulites are of igneous origin^{4,5}, and while those with the higher REE contents and no Eu anomalies may represent near-liquid compositions, the others are likely to be either residua after partial melting or cumulates. The characteristic positive Eu anomalies indicate that plagioclase was an important residual mineral, and the variation in the abundance of heavy REE with relatively little change in Sm/Yb suggests that garnet was not. Moreover, analyses of separated minerals have shown that in xenoliths with the distinctive positive Eu anomalies, all the constituent minerals (garnet and clinopyroxene, as well as plagioclase) display similar anomalies⁹. This emphasizes the metamorphic nature of the present mineral assemblage, implies that the REE patterns were established before granulite-facies metamorphism, and suggests that petrogenetic models should be based on an inferred low pressure (garnet-free, plagioclase-rich) igneous mineralogy. As the low pressure mineralogy of basic rocks is often comparable with their CIPW norms, the following models use normative, and not modal, assemblages.

Various models have been proposed in which the lower crust is residual after large-scale anatexis, and the melt contributes to the more siliceous upper crust^{10,11}. In these models, the lower crust is rich in residual plagioclase and so acquires a positive europium anomaly to balance the negative anomaly characteristic of upper crustal rocks¹⁰. However, such a model does not readily explain the composition of the Lesotho basic granulites. First, they exhibit a relatively restricted range of SiO₂ (42–51%) and those with the higher values tend to have lower REE contents. This is the opposite of what might be expected during intracrustal partial melting when a reduction in SiO₂ is likely to be accompanied by lower total REE contents. Second, during partial melting plagioclase tends to melt more rapidly than any co-existing mafic minerals, and yet the low REE granulites which exhibit the distinctive positive Eu anomalies contain 40–60% normative plagioclase, together with 15–17% olivine and 9–34% clinopyroxene. This compares with <45% normative plagioclase in more REE enriched basic granulites. Finally, the relative variations in compatible and incompatible trace elements (Table 1, and refs 4, 5) appear contrary to that predicted for residua after partial melting. Frey and Prinz¹² have argued that compatible trace elements in such residua will have a limited range in concentration, whereas the abundance of incompatible elements may be highly variable. Their model was based on the extraction of basic melts from the mantle but the principles of their approach can be applied to partial melting within the crust. In this case pyroxene and plagioclase feldspar would be the major residual phases so that Sr would have been compatible and Rb and Nd incompatible. Their concentrations in the residual xenoliths vary by factors of 18, 6 and 6 respectively (Table 2). This is the opposite of that expected in residua

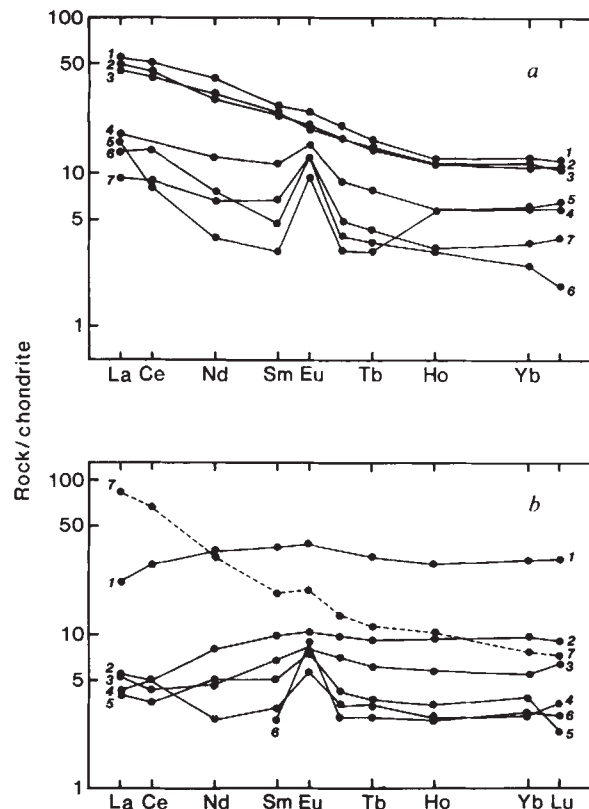


Fig. 2 Chondrite normalized REE abundances for the Lesotho granulite xenoliths. Normalizing values from ref. 24. REE determined by INAA²⁵. a: 1, MAT 12; 2, L 20; 3, PHN 1646; 4, PHN 1919; 5, PHN 2533; 6, PHN 1670; 7, PHN 2852. b: 1, LQ 4; 2, L 12b; 3, L 12a; 4, L 13; 5, OVK F 10303; 6, PHN 2855; 7, LT 2 (felsic granulite).

after partial melting, but it is consistent with a cumulate origin¹² which we shall now consider in more detail.

Trace element abundances of cumulates were calculated using the normative mineralogy of the residual basic xenoliths and assuming that the minerals had crystallized from particular magmas. It is clearly extremely difficult to obtain a realistic value for the amount of intercumulus liquid, so it was decided simply to re-calculate the norms of the xenoliths in terms of plagioclase, olivine and clinopyroxene, and recast all other normative components as trapped liquid and assign them a K_D value of 1. By treating the model in this way, the amount of intercumulus liquid is bound to be underestimated as it will contain some of the normative pyroxene and plagioclase components. Bulk distribution coefficients for these normative assemblages were calculated from published K_D values (REE^{13,14}, TiO₂ and Zr (ref. 15), Sr (ref. 14) and the trace element abundances of the hypothetical cumulates were estimated assuming that the crystals had been in equilibrium with liquids which were either LREE enriched or slightly LREE depleted (for example, L 20 and LQ 4, Fig. 3). Such calculations produce trace element patterns similar to, but slightly lower

Table 1 Rare earth element analyses of granulite xenoliths

	MAT 12	L 20	PHN 1646	PHN 1919	PHN 2533	PHN 1670	PHN 2852	LQ 4	L 12b	L 12a	L 13	F 10303	PHN 2588	LT 2
La	17.8	16.1	14.9	5.74	5.25	4.53	3.07	7.27	1.84	1.76	1.33	1.39	—	27.3
Ce	44.6	38.3	35.2	13.8	7.19	12.4	7.74	24.6	4.32	3.84	3.13	4.29	—	58.4
Nd	25.2	18.9	20.0	8.1	2.33	4.88	4.25	21.5	5.07	2.97	3.15	1.74	—	21.2
Sm	5.46	4.78	4.95	2.30	0.63	0.97	1.39	7.42	1.99	1.38	1.04	0.68	0.58	3.71
Eu	1.93	1.57	1.52	1.20	0.72	0.96	0.97	2.96	0.79	0.65	0.58	0.44	0.64	1.48
Tb	0.88	0.75	0.77	0.40	0.16	0.18	0.22	1.63	0.48	0.33	0.20	0.18	0.15	0.59
Ho	1.00	0.90	0.88	0.45	0.46	—	0.25	2.23	0.73	0.45	0.27	0.22	0.22	0.80
Yb	2.87	2.62	2.49	1.30	1.32	0.56	0.77	6.61	2.11	1.21	0.87	0.67	0.68	1.72
Lu	0.41	0.36	0.37	0.20	0.22	0.06	0.13	1.04	0.31	0.22	0.08	0.12	0.10	0.25

Table 2 Nd- and Sr-isotope results

Sample*	Pipe†	SiO ₂ ‡	Rb‡	Sr‡	⁸⁷ Sr/ ⁸⁶ Sr§	Sm	Nd	¹⁴⁷ Sm/ ¹⁴⁴ Nd	¹⁴³ Nd/ ¹⁴⁴ Nd¶
MAT 12	M	42.9	5	268	0.70408 ± 4	4.73	20.68	0.138	0.512092 ± 14
LQ 4	Li	44.0	21	283	0.70423 ± 5	8.11	27.72	0.177	0.512464 ± 16
PHN 1919	Mo	45.2	14	195	0.70483 ± 3	1.96	7.86	0.151	0.512281 ± 20
PHN 1646	M	45.6	25	1050	0.70396 ± 4	4.30	18.12	0.143	0.512101 ± 14
PHN 2495	Li	47.0	1	163	0.70590 ± 4	7.56	33.60	0.136	0.512265 ± 12
PHN 2852	M	47.1	12	860	0.70412 ± 3	1.30	5.65	0.139	0.512322 ± 16
PHN 1670	M	49.2	30	3556	0.70372 ± 3	0.97	4.94	0.118	0.512307 ± 12
F 10303	M	49.3	8	1250	0.70365 ± 4	0.59	2.23	0.160	0.512570 ± 14
L 12A	M	49.5	11	812	0.70359 ± 3	1.17	3.18	0.223	0.512951 ± 22
L 12B	M	49.7	5	698	0.70376 ± 3	1.52	4.46	0.206	0.512819 ± 10
L 13	M	49.9	10	882	0.70374 ± 4	0.88	2.72	0.196	0.512696 ± 26
M1	M	50.4	13	1081	0.70373 ± 4	1.18	5.12	0.139	0.512232 ± 14
PHN 2533	P	50.4	31	700	0.70373 ± 3	0.59	2.76	0.129	0.512472 ± 18
PHN 2588	Li	51.2	23	625	0.70507 ± 4	0.52	1.30	0.242	0.513019 ± 24
LT 2	L	59.9	3	294	0.70700 ± 4	3.42	20.50	0.101	0.511764 ± 14

* All basic granulites, except PHN 2495 (pyroxenite) and LT 2 (felsic granulite).

† Kimberlite pipes: L, Letseng; Li, Lihobong; M, Matsoku; Mo, Mothae; P, Pipe 200.

‡ Analyses from refs 4 and 5, except MAT 12 which is a new analysis.

§ NBS987 = 0.71015 ± 2. || Isotope dilution, ± 0.5%. ¶ Normalized to ¹⁴⁶Nd/¹⁴⁴Nd = 0.7219, BCR-1 = 0.51262 ± 2.

than, those observed in the low REE granulites (Fig. 3). Moreover, it is very striking that in both the analysed and calculated patterns those with lower REE abundances tend to have larger positive Eu anomalies. The relatively low concentrations in the calculated patterns are probably due to the fact that our model tends to underestimate the amount of intercumulus liquid. Because the latter is enriched in incompatible elements, any underestimate of the amount of trapped liquid will also cause the calculated incompatible element contents of the cumulates to be too low. Such models can only be expected to provide minimum values for the concentrations of incompatible elements in cumulates. Thus the similarities between the observed and calculated trace element patterns, and even the slightly low abundances of the latter, are considered to be strong evidence for a cumulate origin for the igneous protoliths of the basic garnet granulite xenoliths.

Nd and Sr isotopes

Nd and Sr were separated using standard two- and one-column ion exchange techniques, and analysed as metal species on triple and single filament assemblies in a VG 54E mass spectrometer with on-line computer control. Chemical blanks are better than 0.5 ng for Nd and 3 ng for Sr, and the means of repeated analyses of BCR-1 ¹⁴³Nd/¹⁴⁴Nd = 0.51262 ± 2, and NBS 987 = 0.71015 ± 2. The measured ⁸⁷Sr/⁸⁶Sr ratios of the basic granulites are low, 0.7036–0.7051 (Table 2), with all but two being <0.7043. This is consistent with their low Rb/Sr ratios (9 of the 13 analysed are <0.024) but no clear isochron relationship was observed, and two have Rb/Sr ratios which are too high to have been responsible for their low ⁸⁷Sr/⁸⁶Sr in the period of time indicated by the Sm–Nd system. The pyroxenite and felsic granulite have significantly higher ⁸⁷Sr/⁸⁶Sr ratios of 0.7059 and 0.7070, but their Rb/Sr ratios are low (0.006 and 0.01 respectively), suggesting that they too had a more complex multi-stage history.

In contrast the Sm–Nd system is much more informative. The measured ¹⁴³Nd/¹⁴⁴Nd ratios vary from 0.51176 to 0.51302, and 10 of the granulites analysed scatter about an errorchron corresponding to an age of 1,400 ± 100 Myr (MSWD = 28), with an initial Nd ratio slightly higher than that of the Earth, or CHUR (ref. 16), at that time (Fig. 4). The four remaining granulites plot above the errorchron, and because they have the lowest Nd contents of those samples whose ¹⁴³Nd/¹⁴⁴Nd ratios were less than those of typical kimberlites at the time of emplacement¹⁷, the observed scatter probably reflects contamination en route to the surface. The age of 1,400 Myr compares with the model Sr age of 1,300 Myr obtained from the average composition of the seven basic granulites with >800 p.p.m. Sr (Table 2), and represents the

best estimate for the maximum age of the lower crust in Lesotho. Mineral results from similar material include ages of 1,500 and 1,050 Myr from U–Pb on zircons¹⁸, and 1,000 and 714 Myr K/Ar on a mica and hornblende respectively¹⁹; and they encouraged Harte *et al.*¹⁹ to reconsider the possibility that the observed mineral assemblages reflect metamorphic conditions which were present in the late Proterozoic.

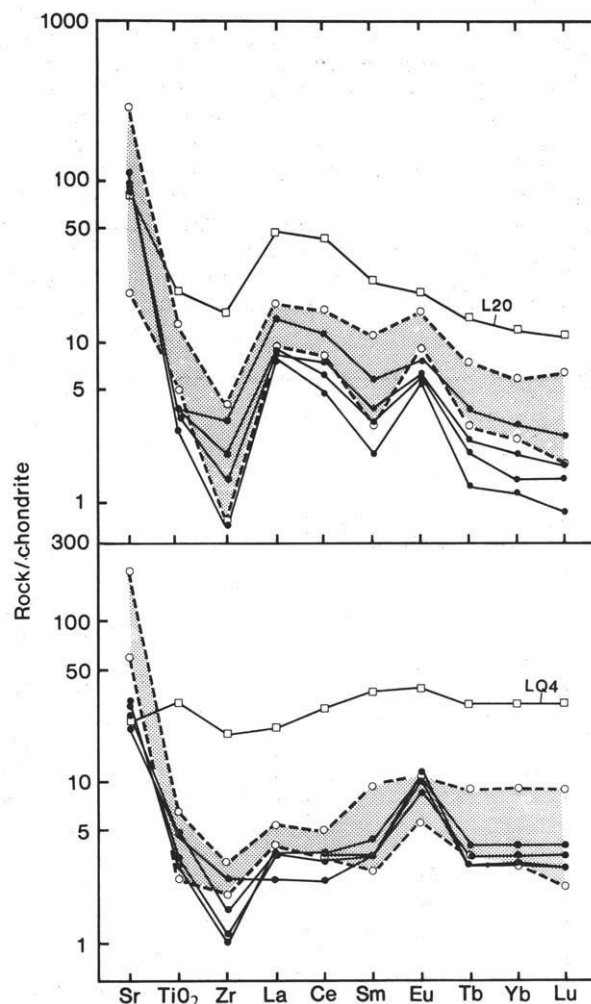


Fig. 3 Comparison of model cumulates (●), based on normative mineralogies, with the xenolith analyses (○). REE normalization values as in Fig. 2, Sr = 11.8 p.p.m., Ti = 620 p.p.m. and Zr = 6.84 p.p.m. □, Liquid compositions used in model.

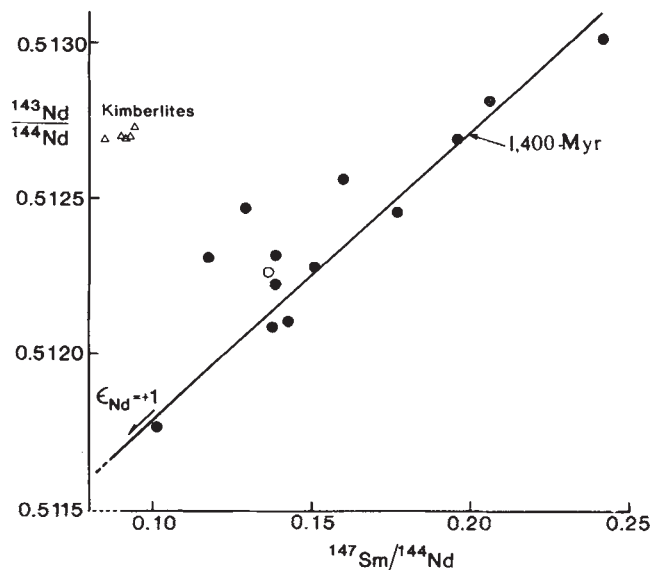


Fig. 4 Sm-Nd isochron diagram illustrating the data on Lesotho granulite xenoliths and representative kimberlites¹⁷ (Δ), O, Pyroxenite PHN 2495.

Discussion

The results presented here suggest that the basic lower crustal xenoliths from Lesotho represent metamorphosed basalts and cumulates which were derived from the upper mantle ~1,400 Myr ago. This Upper Proterozoic age is consistent with the suggestion that granulite xenoliths of this type are characteristic of kimberlite pipes around the edge of the Archaean craton⁴ (Fig. 1), and it would appear in conflict with the idea that diamondiferous kimberlites are unlikely to occur in the Proterozoic mobile belts. However, it has been argued that many of the upper crustal gneisses and metasediments in the Lesotho kimberlites are lithologically similar to those exposed in Archaean terrains²⁰, and it may be that Archaean upper crustal rocks overlie a Proterozoic lower crust in this area.

Cox²¹ has recently argued that most continental flood basalts are probably derived by fractional crystallization from more

picritic parental magmas, and that consequently significant volumes of cumulates may occur within the lower continental crust. One of the best examples of such a volcanic province is the Karoo of southern Africa, and as the Lesotho basic granulites are found in kimberlites which intrude but are some 100 Myr younger than the local karoo volcanics, they were likely candidates for such cumulate material. Moreover, combined Nd- and Sr-isotope work on a variety of Karoo volcanic rocks indicates that many were derived from upper mantle source regions which had been enriched in incompatible elements for almost 1,000 Myr before their eruption at 190 Myr (ref. 22). Hence it might be argued that the granulite Sm/Nd whole rock results (Fig. 4) represent some form of erupted, or source isochron, rather than the time of their separation from the upper mantle. A test of this hypothesis is provided by a plot of ϵ_{Nd} against ϵ_{Sr} in which the initial $^{143}Nd/^{144}Nd$ and $^{87}Sr/^{86}Sr$ ratios of Karoo volcanics are compared with the Nd- and Sr-isotope ratios of the basic granulites at the time of Karoo volcanism, 190 Myr (Fig. 5).

In Fig. 5 a few Karoo volcanic rocks plot on the mantle array, but most fall in the bottom right-hand quadrant which is where rocks (or source regions) with higher Rb/Sr and lower Sm/Nd than the 'bulk Earth' evolve with time. (Note also that 25 of the analysed Karoo rocks have initial ϵ_{Sr} greater than +45 and so plot to the right of Fig. 5.) The basic granulites by contrast have a relatively restricted range in ϵ_{Sr} , and a large variation of ϵ_{Nd} , and so plot in a near vertical trend down into the bottom left quadrant. None of the basic garnet granulites analysed fall with most of the Karoo volcanics in the bottom right quadrant, strongly suggesting that these two rock suites are not genetically related. The two xenoliths which do plot in the bottom right quadrant are the felsic granulite LT 2 (an improbable cumulate), and the pyroxenite PHN 2495 which, on the basis of the arguments presented here, is the one xenolith which might be a cumulate of Karoo age.

Accepting that the Sm/Nd whole rock age (Fig. 4) reflects the time of crystallization of the granulite protoliths then they presumably represent a magmatic episode early in the evolution of the Natal mobile belt. Sr-isotope studies on granites and gneisses within the belt yield ages of 1,000–1,300 Myr and low initial $^{87}Sr/^{86}Sr$ ratios suggesting that any crustal precursors were <1,500 Myr old². If crustal reworking took place it seems

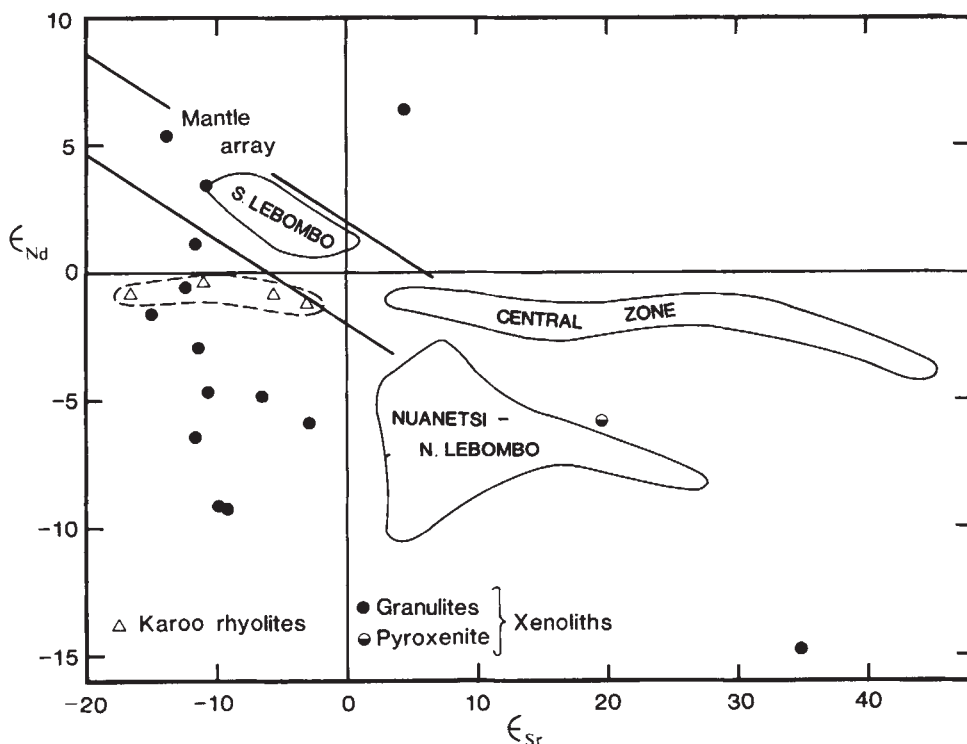


Fig. 5 $\epsilon_{Nd}/\epsilon_{Sr}$ for the Lesotho granulite xenoliths and Karoo volcanic rocks²² at 190 Myr.

only to have involved comparatively young material; there is as yet no indication from either lower crustal xenoliths or surface exposures that significant quantities of Archaean crust were remobilized in this area. However, the recognition that considerable volumes of new continental crust was generated in the period 1,400–1,000 Myr (ref. 2 and this work) raises the intriguing question of whether there is a relationship between that event and the fact that the isotopic variation observed in both Karoo lavas²² and diopsides in garnet peridotites from the Kimberley area²³ reflect chemical variations which may have persisted for ~1,000 Myr. The formation of a belt of new, and probably thicker continental crust around an old cratonic nucleus clearly increases the area of stable continental crust (Fig. 1), and that in turn should increase the volume, and perhaps even the thickness, of mantle material incorporated in

this segment of continental lithosphere. Thus it is envisaged that the formation of the Natal-Namaqua belt may have ultimately stabilized a 'late Proterozoic' lithosphere beneath this area of southern Africa, which was to be sampled some 1,000 Myr later by Karoo magmatism and the xenoliths in kimberlite pipes.

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Serotonin and cyclic AMP close single K⁺ channels in *Aplysia* sensory neurones

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We have identified a serotonin-sensitive K⁺ channel with novel properties. The channel is active at the resting potential; its gating is moderately affected by membrane potential and is not dependent on the activity of intracellular calcium ions. Application of serotonin to the cell body or intracellular injection of cyclic AMP causes prolonged and complete closure of the channel, thereby reducing the effective number of active channels in the membrane. The closure of the channel can account for the increases in the duration of the action potential, Ca²⁺ influx, and transmitter release which underlie behavioural sensitization, a simple form of learning.

SINGLE-CHANNEL current recordings¹ have been used to analyse the properties of ion channels that are directly gated by voltage^{2–5}, intracellular Ca²⁺ (refs 6–9) and chemical transmitters^{10–12}. Previous analyses of synaptic transmission, for example those of the acetylcholine-activated channels at the neuromuscular junction¹³, have focused on synaptic potentials of brief duration where the action of the transmitter leads directly to the opening of a channel coupled to a receptor. These channels are relatively independent of voltage and open only in the presence of transmitter, and so do not normally contribute to the resting or action potential.

Transmitters can also produce slow synaptic actions; often, this does not lead directly to the opening of a channel but modulates the gating of a voltage-dependent channel that contributes to the resting or action potential^{14–16}. Thus, slow synaptic actions can alter the cell's resting and active electrical properties. Alterations in the configuration of the action potential can affect Ca²⁺ influx and modify transmitter release from the neurone presynaptic terminals¹⁷. Furthermore, with some slow synaptic actions, the receptor for the transmitter is often not directly coupled to the channel but to an adenylate cyclase that

activates a series of biochemical steps leading to a cyclic AMP-dependent phosphorylation of the channel or a protein related to it¹⁸.

Serotonin elicits a long-lasting excitatory postsynaptic potential (e.p.s.p.) in certain molluscan neurones^{19–21}. In *Aplysia* sensory neurones it produces a slow e.p.s.p. and facilitates transmitter release²² through a cyclic AMP-dependent phosphorylation²³ that leads to a decrease in a specific K⁺ conductance²⁴. This action of serotonin is thought to underlie behavioural sensitization, an elementary form of short-term memory. The serotonin-sensitive K⁺ conductance differs from the four previously identified neuronal K⁺ currents^{25–27,31}. Using single-channel recording techniques, we have now identified in *Aplysia* sensory neurones a serotonin-sensitive K⁺ channel with novel properties. The channel is active at the resting potential. Its gating is not affected by the activity of intracellular Ca²⁺ ions and is only moderately dependent on membrane potential. Application of serotonin to the cell body or intracellular injection of cyclic AMP causes prolonged and complete closure of the channel. As a result, serotonin appears to reduce the effective number of active channels in the membrane.

Serotonin closes outward current channels

The most frequently observed channel in a membrane patch from a sensory cell gives rise to an outward elementary current of ~ 3 pA at 0 mV. This channel is modulated by serotonin (Fig. 1). In the absence of serotonin (Fig. 1a), the net patch membrane current fluctuates among several discrete levels, reflecting the superposition of the random openings and closings of several individual ion channels that are active in the patch. The unit current steps are, in general, all of a similar amplitude, suggesting that a single type of channel predominates. The probability that a given number of channels is open at any one time follows the binomial distribution, indicating that channels open and close independently of one another. The channels display two types of closures: brief closures lasting only a few milliseconds or less (the closings appear as frequent downward spikes in Fig. 1a), and much longer closures lasting for tens or hundreds of milliseconds (apparent as the step changes in current level).

Application of serotonin caused a dose-dependent increase in the cell input resistance and a reduction in channel activity. As Fig. 1b shows, the addition of ~ 30 μ M serotonin to the bath reduced the number of active channels from five to two. With ~ 60 μ M serotonin in the bath, the remaining channels closed (Fig. 1c) and remained largely closed for the remainder of the drug application (4 min). On superfusion of the ganglion with serotonin-free artificial seawater (ASW), the activity of the channels partially recovered within the next 5–10 min (Fig. 1d).

Serotonin seems to act on individual channels in an all-or-nothing manner (Fig. 1). Channels once closed by serotonin remain closed, while channels which still open in the presence of serotonin seem to open and close normally. Thus, serotonin does not promote the appearance of channels with a reduced current amplitude or channels that open for periods of time that are briefer than normal. Rather, the transmitter causes channels to close for prolonged times (≥ 1 min) and, therefore, on the time scale of normal channel gating, seems to reduce the number of channels active in the membrane patch. Four experiments using fluctuation analysis of single-channel currents²⁸ confirmed that there is no change in the gating kinetics of channels which remain active in the presence of transmitter.

Power spectra of single-channel currents (determined between 1.0 and 1,000 Hz) displayed two lorentzian kinetic components with average time constants of 1 ± 0.1 and 29 ± 8 ms (at 0 to +10 mV). In the presence of serotonin, there was a reduction in total outward patch current but no significant change in the time constants of the two lorentzian components.

In 10 out of 13 experiments, serotonin produced a clear reduction in channel activity. In five of these experiments, serotonin almost completely inhibited channel openings and in the other five it reduced the average activity (defined as average patch current, see Fig. 3c) by 50–90%. In two of the three experiments where it did not affect channel currents, serotonin also had no effect on the cells' input resistance.

Single channel current is voltage dependent

We next examined the effects of membrane potential on current flow through the serotonin-sensitive channels (Fig. 2). The channel contributes outward current and is open for a large fraction of the time, even at potentials negative to the resting level. As the membrane is depolarized, the size of the unit current step increases, although there is only a small change in channel kinetics and in the probability that the channel is in the open state (see Fig. 3c).

The single-channel current-voltage (i - V) relationship displays outward rectification (Fig. 3b). A linear extrapolation of the i - V curve yields an estimate for the reversal potential, E_{rev} , that is negative to -65 mV, suggesting that K^+ is the major charge carrier of this current. Moreover, the i - V curve can be fitted by the Goldman-Hodgkin-Katz²⁹ constant field current equation for a K^+ current. In two experiments the K^+ dependence of current flow through the channel was tested directly. Normal-sized single-channel currents were recorded from cell-free (inside-out)¹ membrane patches where the 'intracellular' surface of the membrane was exposed to a bath solution containing 360 mM K^+ and 10 mM Na^+ . When the solution was changed to normal Na^+ ASW (10 mM K^+), current flow through the channels was dramatically reduced.

The time-averaged current through the serotonin-sensitive channel, $\langle I \rangle$, also displays outward-going rectification (Fig. 3c, open symbols). This largely reflects the voltage dependence of

Fig. 1 Action of serotonin (5-HT) on single-channel current. Patch clamp current recorded from mechanoreceptor sensory neurones in the abdominal ganglion of *Aplysia californica*. Trace a, current in the absence of serotonin. Left-hand ordinate shows the number of open channels; right-hand ordinate shows current magnitude. Individual current steps are 2.6 pA. The current record was well fitted by a binomial distribution assuming that five channels are active in the patch and that each channel opens with a probability of 0.84. Trace b, current obtained 2 min after addition of 30 μ M serotonin to the bath. Trace c, 1 min after addition of a further dose of serotonin, raising the total concentration to 60 μ M. The two traces are a continuous recording. The downward current deflection at the end of the bottom trace is from an intracellular current pulse used to monitor cell resistance. Trace d, current record obtained about 5 min after superfusion with serotonin-free ASW. The small reduction in unit current was probably due to a slight hyperpolarization of the cell resting potential as the intracellular microelectrode was withdrawn from the cell before solution change. The single-channel currents were recorded in response to steady depolarizations of the patch membrane to 0 mV (defined as patch pipette potential minus cell resting potential) produced by changing the potential inside the patch pipette. The use of steady-state recording conditions minimizes interference from inactivating K^+ , Na^+ and Ca^{2+} currents²⁷. In contrast, serotonin-sensitive K^+ current is active during steady-state depolarizations^{26,33}. The bath ASW had the following composition: 470 mM Na, 10 mM K, 10 mM Ca, 55 mM Mg, 600 mM Cl, 10 mM HCO_3^- , 10 mM Tris buffer at pH 7.4. Abdominal ganglia were pretreated with 0.2% trypsin (Sigma, type 1X) in ASW for 10–20 min. Current records were filtered at 1.0 kHz. Temperature, 23 °C.

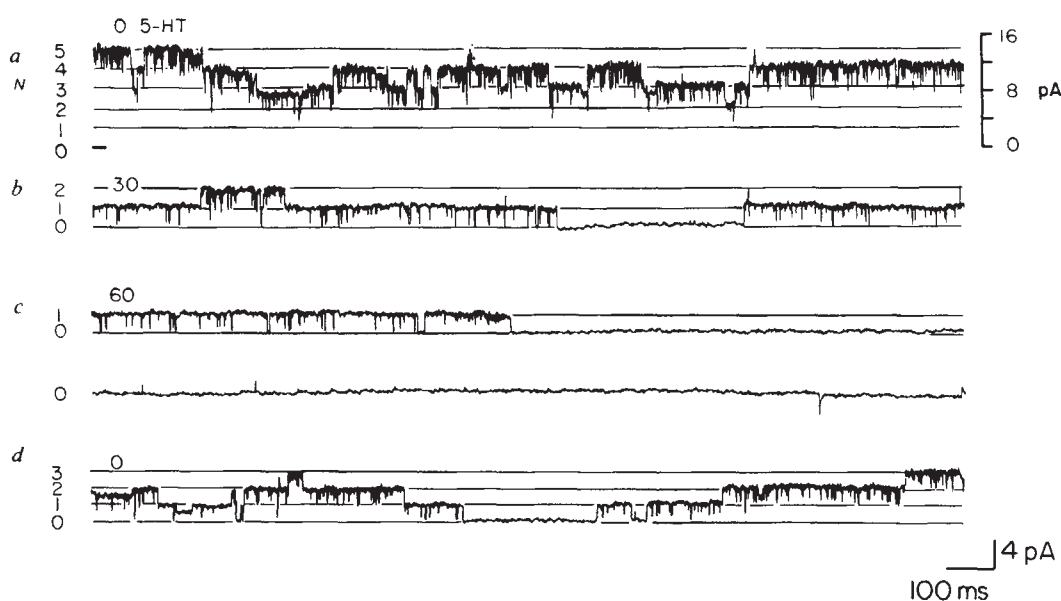
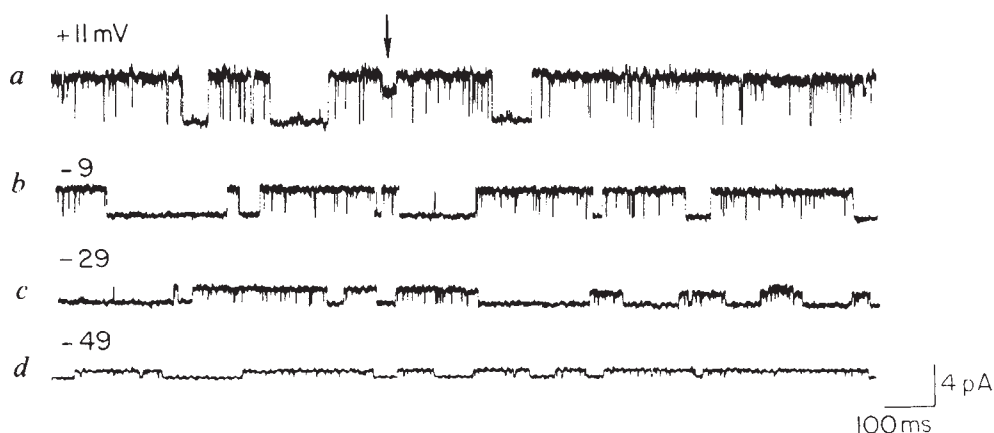


Fig. 2 Single-channel current at different membrane potentials. Current records shown are at four different patch membrane potentials (indicated at the left of each trace). Cell resting potential = -39 mV. Channel openings appear as step increases in outward current. The current fluctuates between two levels corresponding to closed and open channel. At all potential levels the channels show both brief and long closures but spend most of the time in the open state. The arrow indicates a partial closure. Addition of $10 \mu\text{M}$ serotonin to the bath caused the prolonged closure of this channel (not shown). Current records *a*–*c* were filtered at 1.6 kHz, record *d* was filtered at 1.0 kHz. Temperature, 23°C .



the i - V relation, as the probability, P , that the channel is open, obtained from $\langle I \rangle = P_i$, shows only a modest dependence on voltage (Fig. 3c, filled symbols). The voltage dependence of the probability of channel opening varied among different membrane patches. The maximum steepness of the relationship between P and voltage ranged over 25 – 100 mV (average 52 ± 35 mV; $N = 6$) for a two-fold change in P . Some of this variability may be related to the fact that the channels can switch between different kinetic states which may exhibit different degrees of voltage dependence (see Fig. 4). The average value for the single-channel conductance (defined as slope conductance at 0 mV) was 55 ± 6 pS ($N = 12$).

The histogram of channel current amplitudes measured at $+11$ mV from the experiment of Fig. 2 shows a single peak, with a mean channel step size of 3.9 pA, indicating that most opening and closing transitions involve current steps of a fixed amplitude (Fig. 3a). However, channels occasionally show partial closings to an intermediate current level (Figs 2a, 4a,c at arrows; see also ref. 30). The conductance of this partially open state is roughly two-thirds that of the fully conducting state. Such events do not represent the opening of a channel which carries a small inward current because the amplitude of the intermediate current step is increased on depolarization of the membrane (compare Fig. 4a and b at arrows). In addition, no inward channels (of similar amplitude and duration as the intermediate current step) were observed during periods of closure of the serotonin-sensitive outward current channel.

Non-stationary gating of channels

The kinetic behaviour of the serotonin-sensitive channels is quite complex; channels can display a low ($P < 0.1$) probability of being open or show a much higher ($P > 0.5$) opening probability. In the records of Fig. 4 (taken from the same experiment as Fig. 2), the channel was initially closed for most of the time (Fig. 4a,b). It then underwent an abrupt, apparently spontaneous, transition (Fig. 4c) to a state which displayed a much higher open probability and remained in this high open probability state for the next 5 min (during which time the records of Fig. 2 were obtained). At this time serotonin ($10 \mu\text{M}$) was added to the bath and the channel closed. The effect of serotonin does not reflect a switching of the channel back to the low open probability state, as serotonin caused channel closures (mean closed time = 64 s) lasting 300 times longer than the mean closed time observed with the channel in the low open probability state. In patches which display more than one active channel, channels can coexist in both high and low open probability states (see, for example, Figs 1b, 5a), suggesting that the different states do not reflect generalized changes in membrane properties or metabolic state of the cell. Serotonin causes closure of channels regardless of whether they are in the high or low probability state of opening.

Channel is insensitive to intracellular Ca^{2+}

The serotonin-sensitive channel seems to be insensitive to changes in intracellular calcium. In four experiments, tetanic stimulation of the cell, which is known to increase intracellular Ca^{2+} in these cells, did not noticeably alter the probability of channel opening or closing. In addition, intracellular injection of EGTA using hyperpolarizing iontophoretic current pulses (up to 33 nC of total charge into a cell of $\sim 3 \times 10^{-11}$ l volume), had no effect on channel activity. To obtain more direct evidence on the effects of intracellular Ca^{2+} on channel gating, we carried out four experiments in isolated cell-free membrane patches in which the intracellular surface of the membrane was exposed to the bath (inside-out patch). Channel opening and

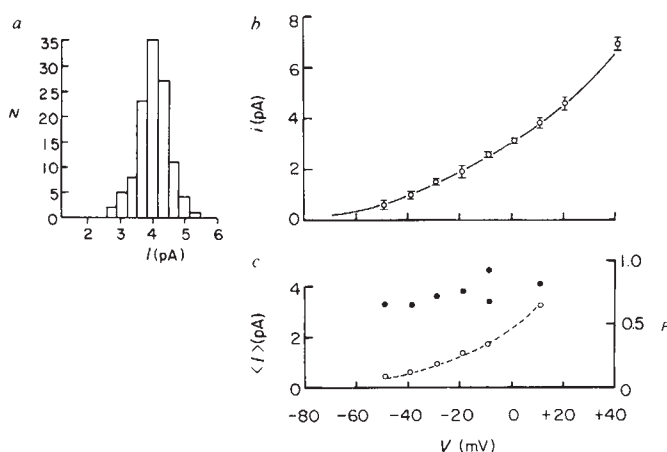
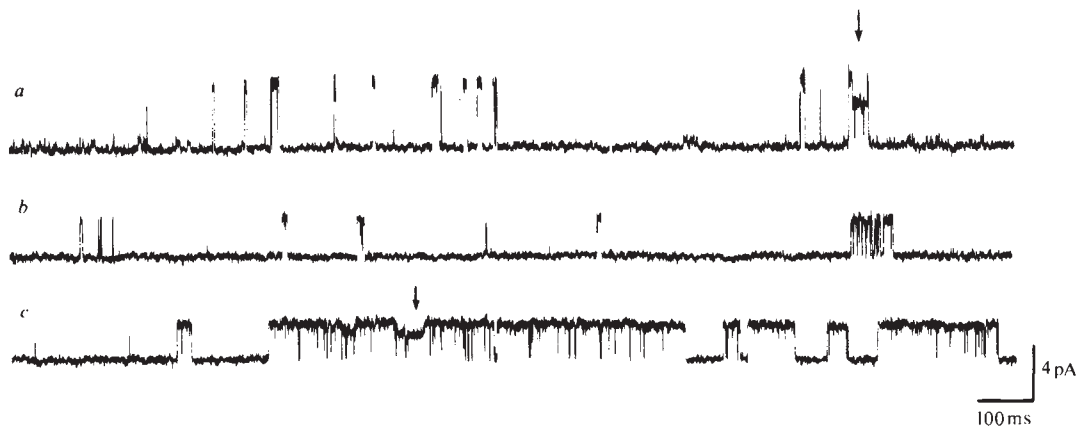


Fig. 3 Voltage dependence of current flow through serotonin-sensitive channel. The three panels are from the same experiment as in Fig. 2. *a*, Histogram of single-channel current amplitudes measured at $+11$ mV. *b*, Single-channel current-voltage relation. Single-channel current amplitudes (i) were measured over a potential range from -49 mV to $+41$ mV and are plotted as a function of membrane potential. The solid curve represents the prediction from the Goldman-Hodgkin-Katz equation for a K^+ current, fitted to the points assuming an intracellular K^+ concentration of 360 mM and a channel K^+ permeability of $8.7 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$. *c*, Current records were digitally sampled at various potentials (at 2.0 kHz sampling frequency) and analysed using an LSI 11/23 computer. Average current flow (I) through the channel was calculated by integrating current flow during channel openings and dividing the integral by the total time of the sample (generally 10 – 20 s of data at each potential). The open symbols show the dependence of $\langle I \rangle$ on membrane potential (left-hand ordinate). Average current is given by $\langle I \rangle = NP_i$, where N is the number of channels in the patch, P is the probability that a single channel is open and i is the amplitude of the single-channel current. Since $N = 1$ in this experiment, $P = \langle I \rangle / i$ (plotted as the filled symbols in *c*, right-hand ordinate).

Fig. 4 Spontaneous transition in channel kinetics. Records show patch membrane current from the same patch as Fig. 2 but slightly earlier in the experiment. *a*, Channel currents at a patch membrane potential of +21 mV. Channels are closed for most of the time. Average probability of channel opening, P , is 0.05. *b*, Channel currents at a potential of -9 mV; $P = 0.025$. *c*, Current record is continuous with the end of trace *b* and shows an abrupt increase in channel open probability; after transition $P = 0.67$. Arrows indicate partial closures.

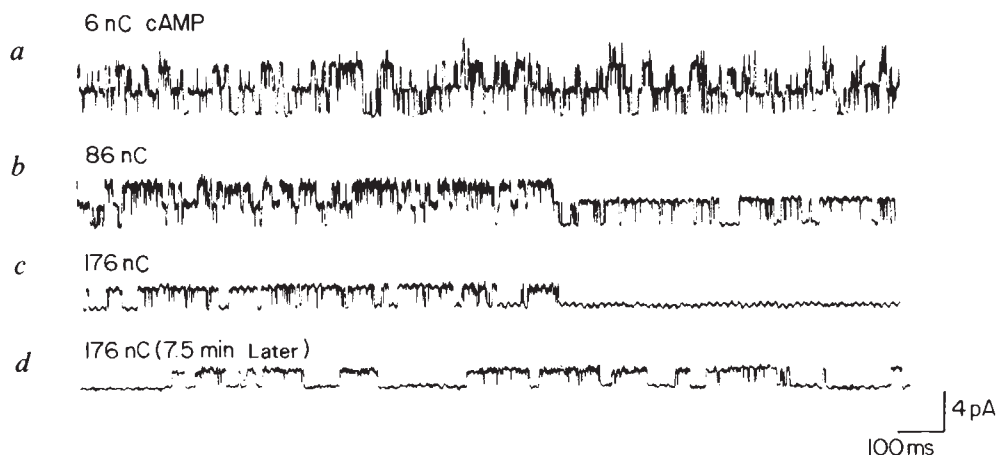


closing were unchanged when the calcium activity at the intracellular face of the membrane was varied from less than 10^{-9} M free Ca^{2+} (0 Ca, 10 mM EGTA) to $\sim 10 \mu\text{M}$ free Ca^{2+} .

Cyclic AMP causes channel closure

The patch pipette forms a very stable and high-resistance seal with the membrane which is thought to prevent diffusion of transmitters or other ligands from outside to inside the pipette. As a result, in the acetylcholine-activated channels of muscle, the transmitter has to be introduced in the patch pipette to affect the channels under the patch pipette¹. By contrast, serotonin consistently decreased channel opening in the patch when applied to the bathing solution outside the pipette. This finding is consistent with the notion that the modulation of the K^+ channel is mediated by means of an intracellular messenger that can act on the patch from within the cell. To test this idea directly, we injected into the cell cyclic AMP, the second messenger thought to modulate the serotonin-sensitive K^+ current (J.S.C. and E.R.K., unpublished observation). Intracellular injection of cyclic AMP, by means of current pulses, causes channels to close in an all-or-none manner similar to the action of serotonin. In the experiment of Fig. 5, three channels are initially active before the start of the current injection (Fig. 5*a*). After the onset of the current pulses, these channels drop out one at a time (Fig. 5*b,c*). Channel activity partially recovered a few minutes after the cyclic AMP electrode had been removed from the cell (Fig. 5*d*). A similar reduction in channel activity on injection of cyclic AMP was observed in four out of six experiments. The effect is not due to the iontophoretic procedure, as similar injections of charge from KCl- or K^+ -EGTA-filled pipettes produced no change in channel gating.

Fig. 5 Effect of intracellular injection of cyclic AMP on single-channel current. Following establishment of the seal between patch electrode and membrane, the cell was impaled with a microelectrode filled with 1 M cyclic AMP (Sigma) and cyclic AMP was injected into cells by hyperpolarizing current pulses (0.5–2.5 nA). Total charge (nanocoulombs) injected into the cell is indicated above each trace. *a*, Current obtained soon after impalement of the cell with the cyclic AMP electrode. *b, c*, Current records after subsequent injection periods. During the record shown in *c* the remaining active channel closed. The cyclic AMP electrode was withdrawn from the cell and no channel openings were observed for 5 min. Channel activity then partially recovered (trace *d*). Cell membrane potential hyperpolarized during the experiment. Patch membrane potential was $\sim +14$ mV in *a*, +11 mV in *b*, +7 mV in *c* and +2 mV in *d*. The time bar in *d* equals 125 ms. Current records were filtered at 1.0 kHz in *a–c* and 800 Hz in *d*. Temperature, 22 °C.



Serotonin-sensitive channels represent a new class of channel

The frequency with which we observe the serotonin-sensitive outward channel suggests that it represents a major K^+ conductance in the cell body of the sensory neurone. The fact that the opening probability of the channels shows moderate sensitivity to membrane potential and no dependence on intracellular Ca^{2+} indicates that the serotonin-sensitive channel is distinct from previously described K^+ currents, including the early K^+ current, delayed rectification, the Ca^{2+} -activated K^+ current and the M current. These findings on the level of single channels are consistent with studies of the macroscopic properties of the serotonin-sensitive current^{24,31}. Because the channel is open at rest and throughout the whole physiological range of membrane potential, the serotonin-sensitive channel may best be described as a background K^+ channel. The inhibition of such a channel is consistent with the physiological effects of serotonin, including depolarization of the resting potential, increase in resting membrane resistance and an increase in the duration of the action potential.

Our results provide direct evidence that serotonin and cyclic AMP modulate the gating of a specific and novel K^+ channel and indicate possible mechanisms whereby this modulation is achieved. The modulatory action does not involve a change in the channel's ionic specificity or detectable changes in single channel conductance or the lifetime of the channel in the open state. Rather, serotonin and cyclic AMP, presumably acting through cyclic AMP-dependent protein phosphorylation, seem to increase the lifetime of the channel in the closed state. This

may simply represent the modification of the normal channel gating process whereby one of the normal rate constants of channel opening is dramatically decreased. Alternatively, serotonin may induce new closed states of the channel (perhaps corresponding to the channel in a phosphorylated form) in which the channel is not available to the normal gating process. In contrast, it has been shown recently that isoprenaline seems to act on the Ca^{2+} channel in heart muscle by increasing the probability that the channel will remain open³².

The ability to record the activity of the serotonin-modulated

channel in cell-free inside-out patches, invites the *in vitro* biochemical study of the steps involved in the cyclic AMP-mediated phosphorylation of single ionic channels. It will be interesting to determine whether the catalytic subunit of the cyclic AMP-dependent protein kinase can phosphorylate the channel in the isolated patch, and whether this phosphorylation can be reversed by a specific phosphatase. These questions are all the more interesting because they lead to a molecular understanding of transmitter modulation that underlies a simple form of learning.

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Genomic environment of *T. brucei* VSG genes: presence of a minichromosome

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Restriction endonuclease maps of some trypanosome variant surface glycoprotein (VSG) genes have a site 3' to the gene where many enzymes appear to cut. This site is sensitive to Bal31 exonuclease, indicating a natural double-strand break in the DNA. One VSG gene exists in several cell clones as a non-integrated minichromosome.

ANTIGENIC variation in *Trypanosoma brucei* involves switching on and off the expression of members of a large family of genes coding for the variable surface glycoproteins (VSGs)¹. In some cases switching of expression of a VSG gene is closely linked to duplication and transposition of the gene^{2–4}. For other VSG genes a similar rearrangement is probably involved in the production of a copy which can be expressed (manuscript in preparation). This copy however, is stable and its expression must be controlled by a mechanism other than the duplication and transposition event itself^{5–7}. Previous work has shown length variations in restriction enzyme fragments extending into 3'-flanking sequences of VSGs genes whose expression is not linked to duplication^{6,8} but similar length variation has also been observed in independently isolated trypanosome clones expressing the same VSG gene whose expression is closely linked to duplication⁴.

We have been studying four VSG genes from the ILTaR 1 antigen repertoire. Of these, only one (ILTat 1.1)⁷ undergoes a rearrangement whose expression appears to be controlled by duplication⁹. There are four distinct copies of the ILTat 1.3 gene, and three of the ILTat 1.4 gene⁶ in each member of a closely related group of trypanosome clones but the number of copies of these genes does not vary with expression. Variation in length of restriction enzyme fragments extending into 3'-flanking regions has been observed for two copies of the ILTat 1.3 gene and one copy of the ILTat 1.4 gene. No rearrangements have been detected in the genomic environment surrounding two copies of each of these genes.

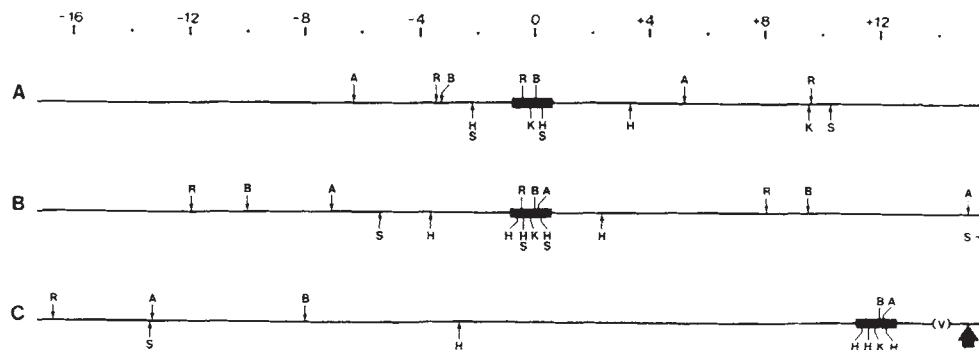
In a comparison of two expressed copies of the *T. brucei* VSG 118 gene, each resulting from different duplication events,

Michels *et al.*⁴ observed a similar variation in the length of 3'-flanking restriction enzyme fragments. However, in this case there were also changes in the length of 5'-flanking fragments. They also reported an apparent cluster of enzyme sites beyond the 3' end of the gene which, they suggested, might be at the end of a chromosome^{4,10}. We have found a similar cluster of enzyme sites beyond the 3' end of those copies of the ILTat 1.3 and 1.4 genes which undergo rearrangement. The cluster is located at the 3'-distal end of the enzyme fragments containing the 3'-flanking sequences. We present here further evidence that these clusters are probably at the ends of chromosomes, and that this location of a VSG gene is not necessarily related to its expression. We also show that one copy of the ILTat 1.3 gene is located near the end of a small linear DNA molecule in native DNA preparations.

Terminal location of VSG genes

The derivation of the ILTaR 1 trypanosome clones used here has been described previously. All clones were derived from a single parent clone (ILTat 1.1) by no more than two relapse infections⁸. Figure 1 shows restriction enzyme maps of the three genomic copies of the ILTat 1.4 gene present in all clones of the ILTaR 1 antigen repertoire so far tested. Restriction enzyme sites internal to the regions of homology with the cloned ILTat 1.4 cDNA (thick bars) were found to be different in the three copies. The sites marked in copy C were identical with those found in the cDNA¹¹. Copies A and B contain an *EcoRI* site not present in the cDNA. In copy A other sites are absent. It therefore seems probable that copy C is the template for transcription of ILTat 1.4 mRNA. The finding of further sequence

Fig. 1 Map of restriction enzyme sites in the vicinity of the three copies of the ILTat 1.4 gene. The scale is in kilobases. The solid bar represents the regions occupied by sequences homologous with the ILTat 1.4 cDNA in plasmids pcBD1 and pcBD2 (J. E. Donelson, J.R.Y. and R.O.W., manuscript in preparation). A = *AvaI*; B = *BglII*; H = *HincII*; K = *KpnI*; R = *EcoRI*; S = *SalI*. (V) indicates a region of variable length in different trypanosome clones. The orientation of the coding sequence (5' to 3') is left to right. The large arrow represents a group of restriction enzyme sites which map to the same position within the accuracy of the measurements made (~200 bp, see Fig. 2). Enzyme sites mapping at this position include *EcoRI*, *AvaI*, *BglII*, *Sau3AI*, *SalI*, *KpnI*, *HincII*, *HaeIII*, *MspI* and *RsaI*. DNA from different trypanosome clones was digested completely with one or more enzymes and used in Southern transfer experiments as described elsewhere⁶, using 5' and 3' cDNA probes from either side of the central *BglII* site (J. E. Donelson, J.R.Y. and R.O.W., manuscript in preparation). The results of double and triple digestion experiments allowed the allocation of particular 5' and 3' fragments to the appropriate genomic copy. Where cloned genomic DNA was available, this allocation was confirmed by analysis of these genomic clones (J. E. Donelson, J.R.Y. and R.O.W., manuscript in preparation). Differences in restriction enzyme sites within the cDNA region were also revealed in these experiments; for example, the 5' *HincII* fragment of copy A also hybridized to the 3' probe, showing that the 5' two *HincII* sites were absent from this copy.



differences between the cDNA sequence and cloned fragments of copies A and B supports this conclusion (J. E. Donelson, J.R.Y. and R.O.W. manuscript in preparation).

For copies A and B in Fig. 1, no differences were detected in their flanking sequences in eight trypanosome clones, three expressing and five not expressing ILTat 1.4. In comparing expressing and non-expressing clones, no differences were found in enzyme sites on the 5' side of the copy C gene in a region of 25 kilobases (kb). This implies that any transposition involved in modulating the expression of this gene would have to involve a transposed section much greater than that observed for other VSG genes¹⁰.

The large arrow in Fig. 1 on the 3' side of copy C represents a cluster of enzyme sites that cannot be resolved within the accuracy of the mapping technique used. Figure 3 illustrates the proximity of these sites for six restriction enzymes, in one trypanosome clone. Each track of this gel contains a hybridizing fragment between 5.5 and 5.9 kb which is known from mapping data to come from the 3' end of copy C. There is a recognition site for each of these enzymes between 75 (*MspI*) and 472 (*SalI*) base pairs (bp) from the 5' end of the pcBD1 cDNA insert (J. E. Donelson, J.R.Y. and R.O.W., manuscript in preparation). The 5.9 and 5.5 labels indicate the size of the *MspI* and the *SalI* fragments determined from measurements on this and other autoradiographs, using the size markers shown. The difference of 400 bp between 3' fragments with these two enzymes is exactly as expected from the position of the sites in the cDNA if the 3' distal sites are at identical positions. The fragments found with the other enzymes are of intermediate length, showing that their 3' ends also map at the same position. Double-digestion experiments like those shown on the right in Fig. 2 did not reveal any site mapping even closely inside the apparent cluster. This experiment resolved differences of less than 200 bp, making this the upper limit on the length of the cluster. Significantly, we did not find a site for any enzyme mapping beyond the position of the cluster. These data suggest that the 'cluster' is in fact a natural double-strand break in the trypanosome DNA. The region of the genome between this break and the gene is unusual in that none of the enzymes we used recognized a site within this region.

We have found a similar cluster of restriction enzyme sites distal to the 3' ends of the two copies of the ILTat 1.3 gene which are subject to 3' length variation (manuscript in preparation). To rule out the possibility that the clustering of enzyme sites was coincidental, we tested the susceptibility of the flanking regions of the ILTat 1.3 gene in intact ILTat 1.3 DNA to the double-stranded exonuclease *Bal31* (ref. 12). Intact DNA was treated with *Bal31* for varying times and then cut with *AvaI*. The nearest *AvaI* sites in the flanking regions of the ILTat 1.3 genes have been mapped and are shown in Fig. 3. The 3' fragments of copies C and D (12.0 and 7.2 kb) are the fragments for which length variation is observed in different trypanosome clones, and at the end of which is the cluster of enzyme sites. The top of Fig. 3 shows that pretreatment with *Bal31* results in progressive shortening of these two fragments while the other fragments are unaffected. The 80-kb band is faint because of shearing in this DNA preparation. This band is the 5' fragment of copy D. Its intensity decreases, but with such a large fragment it is not possible to distinguish between exonucleolytic shortening and cleavage by any contaminating endonuclease. This experiment demonstrates that the ends of the 3'-flanking

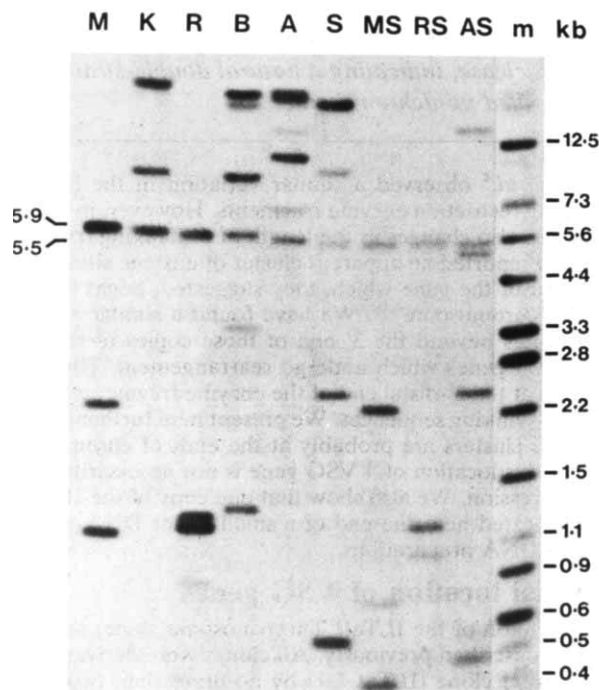


Fig. 2 Illustration of the close mapping of restriction enzyme sites distal to the 3' end of copy C of the ILTat 1.4 gene. DNA from trypanosome clone ILTat 1.2 was completely digested with restriction enzymes M = *MspI*, K = *KpnI*, R = *RsaI*, B = *BglII*, A = *AvaI* and S = *SalI*. Tracks MS, RS and AS are double digests with the indicated enzymes. Fragments were resolved by electrophoresis in a 0.75% agarose gel, transferred to nitrocellulose and hybridized to pcBD1 as described elsewhere⁶. Molecular weight markers (m) are various fragments of known length hybridizing to pBR322.

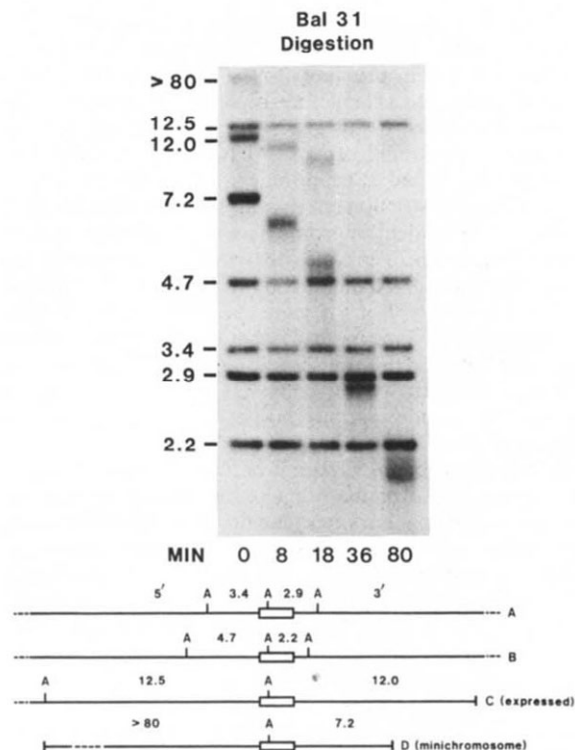


Fig. 3 *Bal31* digestion of nuclear DNA. 40 μ g of nuclear DNA was digested with 13 units of *Bal31* enzyme in 600 mM NaCl, 12 mM CaCl_2 , 12 mM MgCl_2 , 20 mM Tris-HCl pH 8 and 1 mM EDTA. The reaction was incubated at 30 °C and aliquots were taken at the times shown by pipetting into water-saturated phenol. Each DNA was precipitated with ethanol and subsequently digested with *Ava*I restriction endonuclease. The digested DNAs were electrophoresed in a 0.8% agarose gel and transferred to nitrocellulose paper as described previously⁶. Preparation of plasmid pcBC1 containing the complete coding sequence for VSG ILTat 1.3 has been described elsewhere⁶. The plasmid was nick-translated and hybridized as described elsewhere⁶. The upper portion of the figure shows the results of the blotting experiments. The lower portion shows a map of the *Ava*I sites in the vicinity of the four copies of the ILTat 1.3 genes.

sequences of copies C and D are accessible to exonuclease digestion in intact DNA. This observation cannot be explained by random breakage as this should affect all fragments equally. It could be the result of a site-specific cleavage by a trypanosome endonuclease during DNA isolation, or the result of a specific region of DNA being unusually susceptible to shearing. Site-specific cleavage of DNA has not been observed in trypanosomes. We are not aware of any DNA structures hypersensitive to shearing. Therefore we prefer the simpler explanation that the restriction enzyme site clusters observed in our work are in fact the site of natural double-strand breaks in the trypanosome genome, and are probably the ends of chromosomes. In ILTat 1.4 DNA, the same two fragments of the ILTat 1.3 gene are susceptible to *Bal31* (data not shown), so that telomeric location of this gene is not correlated with expression.

A VSG gene on a minichromosome

The observation of two copies of the ILTat 1.3 gene, each adjacent to the end of a DNA molecule, led us to investigate whether these genes might be located on unusual extrachromosomal DNA structures similar to the small non-integrated pieces of DNA which carry the ribosomal RNA genes in the macronucleus of *Tetrahymena*¹³. To test this possibility minimally sheared DNA was prepared from three trypanosome clones expressing VSGs ILTat 1.2, 1.3 and 1.4 and fractionated on sucrose density gradients. Figure 4 shows the distribution of the four copies of the ILTat 1.3 genes in these gradients. *Hinc*II

does not cut the gene and the single *Hinc*II fragment corresponding to each gene copy in these clones is known from restriction enzyme mapping data (manuscript in preparation). We reported previously that the ILTat 1.3 clone contains a minor cell population in which copy D of the ILTat 1.3 gene is on a different-sized *Hinc*II fragment⁶. This is the larger fragment seen in fraction 16 of the ILTat 1.3 gradient and is smaller than the copy C *Hinc*II fragment seen in fractions 20 to 30. In all three clones the DNA carrying copy D was clearly separated from the bulk of the DNA which was sedimented to the bottom of the tube. Copy D is located on a DNA molecule that is not integrated into, and is smaller than, the bulk of

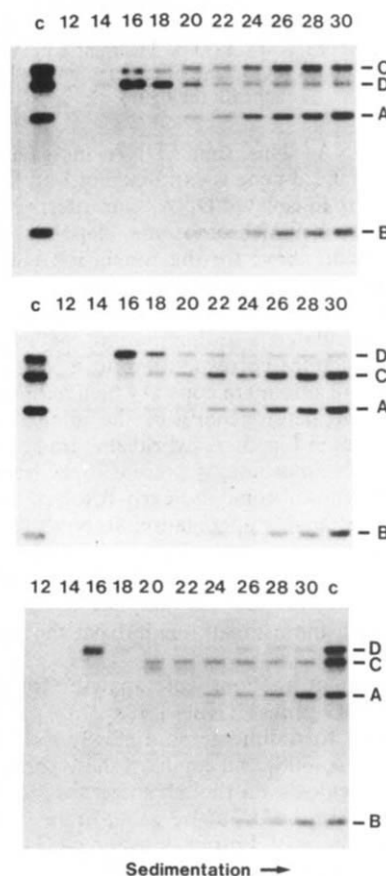


Fig. 4 Location of ILTat 1.3 genes in fractions from intact trypanosome DNA sedimented through a sucrose density gradient. Trypanosomes homogeneously expressing different VSGs were isolated by Percoll density gradient centrifugation¹⁸, and suspended at $2-4 \times 10^8$ cells per ml in 25 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 8.0 (TNE) in 30-ml centrifuge tubes. Trypanosomes were lysed by addition of SDS to 0.5%. The lysate was incubated for 2 h with 100 mg ml^{-1} RNase A (previously heated to 70 °C for 60 min), then for 4 h with 1 mg ml^{-1} of Pronase (preincubated at 37 °C for 2 h), both at 37 °C. The lysate was extracted with an equal volume of phenol (redistilled, saturated with TNE) in 125-ml conical flasks in a giratory incubator at ~60 r.p.m. at room temperature overnight. The aqueous phase was transferred to 30-ml centrifuge tubes and centrifuged at 5,000 r.p.m. for 10 min in a Heraeus-Christ minifuge. The extremely viscous DNA solution ($10-20 \mu\text{g ml}^{-1}$) was transferred with a wide-mouthed pipette tip into dialysis tubing and dialysed against four changes of 50 volumes of TNE. 5 ml of the dialysed DNA was layered onto a 10–40% sucrose gradient in 10 mM Tris-HCl, 5 mM EDTA pH 8.0 and centrifuged at 24,000 r.p.m. for 10 h in a Beckman SW27 rotor at 20 °C. The gradient was fractionated with an ISCO gradient fractionator (30×1.2 -ml fractions). The DNA was precipitated by addition of NaCl to 0.2 M and 2.5 volumes of ethanol, recovered by centrifugation, washed in 75% ethanol and resuspended in 10 M Tris-HCl, 0.1 mM EDTA pH 8.0. The position of the ILTat 1.3 genes in the gradient was determined by a Southern blot analysis of DNA from alternate fractions digested with *Hinc*II, using pcBC1 as a probe. Direction of sedimentation was from left to right. Tracks marked C are digests of unfractionated DNA. Top, clone ILTat 1.3; middle, clone ILTat 1.4; bottom, clone ILTat 1.2. A, B, C and D mark the *Hinc*II fragments from the four distinct copies of the ILTat 1.3 gene (manuscript in preparation). The gradient of the ILTat 1.2 DNA contained half the amount of DNA used in the others reducing the extent of trailing due to aggregation.

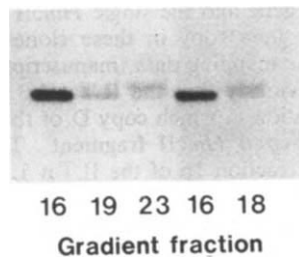


Fig. 5 Electrophoresis of intact minichromosome DNA. DNA recovered from the gradients shown in Fig. 4 was electrophoresed in a 0.55% agarose gel at 1 V cm^{-1} for 40 h. The DNA was transferred to nitrocellulose, and hybridized with ^{32}P -labelled pcBC1 as described elsewhere⁶. The three left-hand fractions were from the gradient of ILTat 1.3 DNA. The right-hand two fractions were from the ILTat 1.4 gradient. Fraction numbers are indicated beneath the figure.

trypanosome DNA. This small DNA molecule is present whether the ILTat 1.3 gene is expressed or not. This molecule is a discrete entity in isolated DNA. Our interpretation, that it exists *in vivo* as a minichromosome, depends on the same arguments as made above for the conclusion that the gene is located next a natural double-strand break.

In all three gradients copy D is located in fractions 16 and 17. We have calculated a sedimentation coefficient ($s_{20,w}$) of 41S for these fractions corresponding to a length of $\sim 80 \text{ kb}$ (ref. 14). The small amount of copy D which sedimented further is not due to size heterogeneity of the minichromosome as shown by the data in Fig. 5. A hybridizing fragment, the same size as the minichromosome, is present in all fractions in this figure. This minichromosome is clearly resolved from the bulk of the DNA. Presumably aggregation accounts for the trailing of the minichromosome peak. By comparison with partially cohered λ bacteriophage DNA we have estimated the minichromosome size to be just less than two λ DNA molecules, which agrees with the estimate made from the sedimentation coefficient (80 kb).

The same gradient fractions were analysed for the presence of three other VSG genes (ILTats 1.1, 1.2, 1.4). None of these genes was found to sediment more slowly than the bulk of trypanosome DNA (80S). All copies of these genes are on very large DNA molecules even though some are located near the ends, as is copy C of the ILTat 1.3 gene. In the gradient of the ILTat 1.2 DNA, the pcBC1 probe detected two faintly hybridizing fragments in slowly sedimenting DNA (fractions 16 and 20). These bands are barely visualized in unfractionated DNA (track C). The pcBC1 probe contains sequences which hybridize to the 3' ends of other VSG genes⁶. These bands may result from different VSG genes on other small DNA molecules. Fraction 20 corresponds to a sedimentation coefficient of 53S

(160 kb). A proportion of molecules carrying copy C also appears to sediment more slowly than copies A and B. This may be a result of its proximity to the end of the chromosome, which would lead to a smaller average size of the sheared DNA in which it appears. If this gene were also located on a minichromosome we would expect to have found a definite peak rather than the broad distribution evident in this gradient.

A number of restriction enzyme sites in and around the ILTat 1.3 gene in the minichromosome have been mapped. These are shown in Fig. 6. All 20 enzyme sites within the gene are identical with sites in the cDNA and in copy C. Five sites 5' of the gene, and the *Ava*II site 3' of the gene are also identical with sites around copy C. It appears that either copy C or D could serve as the template for the transcription of mRNA. However the preferential sensitivity of copy C to DNase I digestion (manuscript in preparation) suggests that copy C is the template in clone ILTat 1.3.

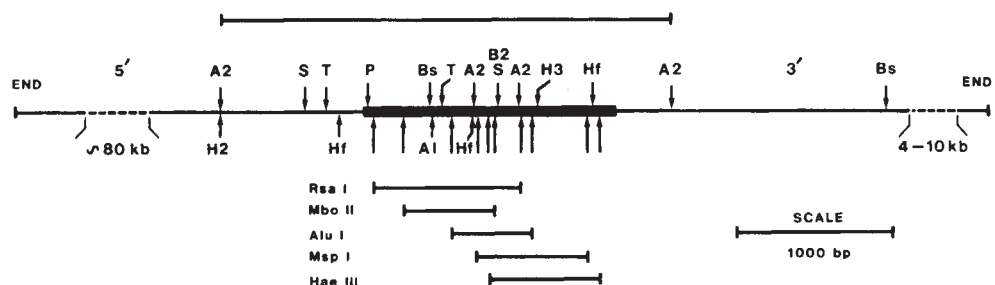
The region of length variation between the *Bst*EII enzyme site and the 3' end of the minichromosome is not cut by 14 different restriction enzymes, including 4 that have 4 base recognition sequences. This suggests that this region is composed of a repetitive sequence, similar to the region adjacent to expression-linked copies of other VSG genes¹⁰ and copy C of the ILTat 1.3 gene. This characteristic region in the 3'-flanking sequences represents another similarity between the minichromosome copy and the expressed copy C.

We have not detected sites 5' of the gene in the minichromosome for 10 different restriction enzymes. For the enzymes *Eco*RI, *Eco*RV, *Bam*HI and *Sal*I, prolonged electrophoresis was used to detect cutting of the intact minichromosome. We would have detected any reduction of more than 20 kb in the length of the minichromosome. Thus we have found no sites for these enzymes more than 20 kb from the 5' end of the minichromosome. For the other six enzymes, listed in Fig. 5 legend, the very large fragments from the 5' end of this gene copy are not detectably shorter than the intact minichromosome. However, the shorter electrophoresis used in these experiments only allows us to conclude that there are no sites less than 40 kb from the gene. Even a region of 40 kb in length uncut by any of these 10 enzymes is remarkable. It seems likely, therefore, that the central region of the minichromosome is also composed of a highly repetitive sequence.

Discussion

Borst *et al.*¹⁵ found several kinds of large DNA molecules after digestion of *T. brucei* DNA with *Hae*III enzyme. These consisted mainly of long linear DNA molecules, but also some circles and lariat-shaped molecules with duplex stems. Because the DNA was cut with *Hae*III, these molecules may have been cut from intact DNA by the enzyme, or may have been present before digestion as discrete molecules. Fractions containing

Fig. 6 Restriction enzyme map of the minichromosome copy of the ILTat 1.3 gene. The thickened bar represents the region of homology with the cDNA in pcBC1. The sites marked by letters were located by Southern hybridization experiments with whole DNA, isolated minichromosome, and cloned genomic DNA from copies A and B, using 5' and 3' probes. The bars beneath the map indicate internal fragments which are of identical size in this copy, copy C and the cDNA. Copy D *Hinc*II fragments from a clone expressing and a clone not expressing ILTat 1.3 were separated by sucrose gradient centrifugation. Southern blot experiments with the enzymes shown at the left of the bars demonstrated the presence of these fragments in both copies. The longer arrows show the position of the ends of these fragments in the cDNA sequence¹¹. The bar above the map spans a region where all enzyme sites are identical in both the minichromosome and copy C (manuscript in preparation). All labelled sites within the region of homology with the cDNA are identical to those in the cDNA. The broken line 5' of the gene represents a region of DNA ($\sim 80 \text{ kb}$) that is not cut by *Eco*RI, *Ava*I, *Msp*I, *Bgl*II, *Eco*RV, *Bst*EII, *Hind*III, *Hgi*AI, *Bam*HI or *Sal*I. The broken 3' of the gene is a region of variable length in different ILTat 1 trypanosome clones. This region is not cut by *Ava*I, *Bgl*II, *Sau*3AI, *Hha*I, *Hae*II, *Pst*I, *Eco*RI, *Sal*I, *Hinc*II, *Bst*NI, *Bam*HI, *Rsa*I, *Eco*RV or *Hind*III. Enzyme labels are A1 = *Ava*I, A2 = *Ava*II, B2 = *Bgl*II, Bs = *Bst*EII, H2 = *Hinc*II, H3 = *Hind*III, Hf = *Hinf*I, P = *Pst*I, S = *Sau*3AI, T = *Taq*I.



these molecules were shown to have a 180-bp *AluI* repeated sequence. The repeated sequence was also frequently cut by *HindIII* as its recognition sequence is related to *AluI*. Since the central region of the minichromosome is not cut by *HindIII*, it appears unlikely that it contains the same repetitive element. As we have demonstrated *Bal31* sensitivity at only one end of the minichromosome, we cannot rule out the possibility that it is one of the lariat molecules observed by Borst. If so this would change the estimated size of the minichromosome.

We have found a minichromosome copy of the ILTat 1.3 gene in all ILTaR 1 clones investigated. The minichromosome appears to be a more stable component of the trypanosome genome than the expression-linked copies of some VSG genes^{3,4}. The ILTat 1.3 VSG gene in the minichromosome is identical to the expressed gene, by restriction enzyme mapping, over a range of at least 2.7 kb, and is similarly situated close to the 3' end of a DNA molecule. Both of these copies (C and D) have probably arisen by mechanisms similar to the generation of expressed copies of other VSG genes (manuscript in preparation). This involves recombination within the 3' end of the coding sequence⁹. The fact that both copies appear to be recombined with the same 3' end, unlike independently duplicated expression-linked copies, suggests that they were both derived from a single recombination event. It may be that one

gene is the precursor of the other. If the minichromosome copy is the precursor of the expressed copy it suggests a role for this unusual structure. The minichromosome may be an intermediate in the production of expressed copies of VSG genes. If this is the case, then our failure to find such a minichromosome copy of other VSG genes suggests that the stability of this particular one is unusual.

There are alternative possibilities. It is interesting to note that the ILTat 1.3 VSG is expressed in metacyclic parasites from the ILTaR 1 antigen repertoire (J. D. Barry, personal communication). Metacyclic VSGs are expressed in the tsetse fly in conditions very different from the blood stream of the mammalian host. It is possible that metacyclic VSG gene expression is controlled by a different mechanism. The minichromosome could be involved in such a mechanism.

Genetic recombination in trypanosomes has been implied from the results of Tait¹⁶. The minichromosome may be a structure involved in intercellular genetic exchange. Of course, until our observations are extended to other VSG genes, it remains possible that the minichromosome is the product of an unusual process within this trypanosome stock.

The success of the trypanosome in presenting the molecular biologist with unusual and interesting phenomena¹⁷ nearly equals its success as a parasite.

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Stereochemistry of cooperative effects in fish and amphibian haemoglobins

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The low oxygen affinity of many fish haemoglobins at low pH is suggested to be due to the replacement by serine of the reactive cysteine F9 β found in mammalian haemoglobins. Model building shows that hydrogen bonds between this serine and the C-terminal histidine stabilize the quaternary deoxy(T) structure. A stereochemical model for the binding of the allosteric effectors ATP or GTP is also advanced.

LACTIC ACID, secreted into the blood by a gland attached to the swim bladder, causes teleost fish haemoglobins to discharge oxygen into the bladder, and thus to raise the buoyancy of the fish¹. This acid-activated discharge is known as the Root effect, but in fact it represents no more than an enhanced version of the alkaline Bohr effect exhibited by mammalian haemoglobins. Both effects consist in a reciprocating action of protons and oxygen: uptake of protons causes release of oxygen and vice versa. They arise because all vertebrate haemoglobins are in an equilibrium between two structures: the deoxy or tense (T) structure and the oxy or relaxed (R) structure. The former has a low affinity for oxygen and a high one for protons, chloride, organic phosphate and CO₂. In the latter these relative affinities are reversed. The factors that affect the oxygen affinity are known collectively as heterotropic ligands. Structural analysis

has shown that they stabilize the T structure by forming salt bridges within or between the subunits. These salt bridges are absent in the R structure². The transition between the two structures in the course of oxygen uptake gives rise to the cooperativity of oxygen binding. Hill's coefficient *n* provides a measure of the degree of cooperativity.

The response of fish haemoglobins to heterotropic ligands is markedly different from that of most mammalian haemoglobins³. While in human haemoglobin *n* remains at or just below 3.0 independent of pH, in fish haemoglobins it drops to unity, or even below unity, near pH6. This disappearance of cooperativity at acid pH is due to inhibition of the allosteric transition from the T to the R structure and is now widely used as a diagnostic of the Root effect. *n* rises to a maximum of only just above 2.0 near neutral pH and in many, but not all fish,

Table 1 Important Amino-Acid Replacements Distinguishing Mammalian, Amphibian and Fish Haemoglobins

Position of amino acid residue†	Human	Rat II and III	Carp	Trout IV	Trout I	Aquatic frog <i>Xenopus</i>	Terrestrial frogs <i>R. esculanta</i> (Europe)	<i>R. catesbeiana</i> (America)	Tadpole of <i>R. catesbeiana</i>	Shark (<i>Heterodontus portusjacksoni</i>)	Lungfish (<i>Lepidosiren paradoxa</i>)
β chain											
NA2	His	His	Glu	Asp	Glu	Gly	—	—	His	His	His
EF6	Lys	Lys	Lys	Lys	Leu	Lys	Lys	Lys	Lys	Lys	Lys
F6	Glu	Glu	Val	Val	Glu	Lys	Glu	Glu	Glu	Lys	His
F9	Cys	Cys	Ser	Ser	Ala	Ser	Ser	Ser	Ala	Ala	Ser
FG1	Asp	Asp	Glu	Glu	Asn	Glu	Asn	Gly	Asn	Glu	Glu
H21	His	His	Arg	Arg	Ser	His	Lys	Lys	His	Lys	Arg
HC1	Lys	Lys	Gln	Gln	Arg	Gly	Ala	Gly	Ser	Glu	Glu
HC3	His	His	His	His	Phe	His	His	His	His	His	His
α chain											
NA1	Val	Val	Ac-Ser	Ac-Ser	Ac-Ser	Leu	Gly	?	Ac-Ser	Ac-Ser	Met-Arg
C3	Thr	Thr	Gln	Gln	Gln	Lys	?	?	Gln	Ala	Gly
Root effect	—	?	+	+	—	+	—	—	—	—	—
Allosteric effector	DPG	DPG	ATP	ATP	—	DPG	?	?	?	?	ATP
Bohr effect	Normal	Normal	Strong	Strong	Absent	Normal	Weak	Weak-Normal	Reversed	Weak	Normal
Refs	40	40–42*	43	44	45	46†	47	48, 49	50, 51	52, 53	54

* Also L. M. Garrick and M. D. Garrick, unpublished.

† Also R. M. Kay, R. Harris and J. G. Williams, unpublished.

‡ For meaning of the symbols see Figs 3–5.

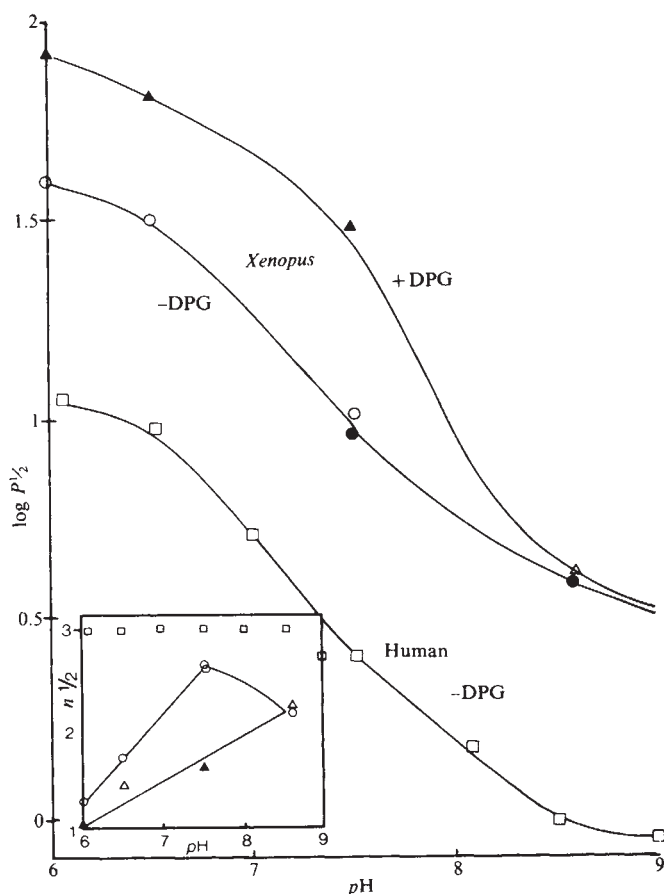


Fig. 1 Oxygen Bohr effect from two different preparations (open and closed symbols) of *Xenopus* haemoglobin at 20 °C. ○, Stripped in 0.1 M bis-Tris or Tris-HCl+0.1 M NaCl. △, Same+5 mM DPG. Haemoglobin concentration 180–300 μ M haem. The insert shows the pH dependence of $n_{1/2}$. Blood from various animals was pooled and washed with 0.7% NaCl. The packed cells were lysed with distilled water, and stroma and nuclei were spun down. The haemoglobin was stripped as described in ref. 55 and dialysed against 0.1 NaCl and water. The haemolysate was analysed by starch gel electrophoresis at pH 8.6 before and after incubation with 2 mM mercaptoethanol, but no evidence of polymerization was found. Oxygen dissociation curves were determined spectrophotometrically. □, Stripped human haemoglobin in 0.05 M bis-Tris+0.1 M NaCl.

falls again at higher pH. Both the span of oxygen affinities covered by the pH range 6–9 and the magnitude of the alkaline Bohr effect ($\Delta \log p_{50}/\Delta \log \text{pH}$) are larger in fish than in mammalian haemoglobins. For instance, in carp haemoglobin k_1 , the association constant of the first oxygen molecule to be taken up, rises 100-fold between pH 6.5 and 9, compared to eight-fold in human haemoglobin; over the same pH range k_4 , the association constant of the last oxygen, rises 10-fold in carp compared to 1.2-fold in human. On oxygenation, human haemoglobin in deionized water releases no more than one proton per tetramer, while carp releases 3.6. On the other hand, the enhancement of the alkaline Bohr effect by chloride or phosphate is smaller in carp than in human haemoglobin. Most mammalian red blood cells contain 2,3-disphosphoglycerate as an allosteric effector, while those of fish contain ATP or GTP instead^{4–11}.

What makes the allosteric properties of these fish haemoglobins so different from those of mammalian haemoglobins? Seeing that the fraction of amino acid replacements between, say, human and carp haemoglobin is of the order of 50%, this seems a forbidding problem, but experience has shown that the great majority of replacements between species is functionally neutral, while a few in key positions dominate the allosteric functions. At a meeting on allostery at Cambridge in August 1980, these thoughts led Perutz to suggest that a single replacement, namely that of cysteine F9(93) β in mammalian haemoglobins by serine in fish, may be sufficient to explain most of the Root effect. The recent discovery of serine in the same position of *Xenopus* haemoglobin then led him to predict that it should also exhibit a Root effect. The results reported below confirm this. Model building and study of amino acid sequences now lead us to propose a detailed stereochemical mechanism for the cooperative effects in fish and amphibian haemoglobins.

Ligand binding by *Xenopus* haemoglobin

Xenopus haemoglobin has a range of oxygen affinities below those of human haemoglobin, suggesting greater stability of its T structure. We have found its alkaline Bohr to be similar in magnitude to that of human haemoglobin, but its Hill's coefficient to approach unity at pH 6.0, as in fish haemoglobins that exhibit the Root effect, suggesting that acid pH inhibits the transition from T to the R structure (Fig. 1). This inference is confirmed by the kinetics of carbon monoxide recombination after flash photolysis. In mammalian haemoglobins this reaction accelerates as combination of successive CO molecules is accompanied by the T→R transition, but in fish haemoglobins at pH 6.0 that acceleration is absent, because the T→R transi-

tion is inhibited⁶. In *Xenopus* haemoglobin the kinetics of CO recombination were obscured at first by partial dissociation into non-cooperative, fast-reacting dimers. We therefore determined the dependence of the fraction of dimers on protein concentration, pH and organic phosphates and corrected our measurements of the time course of recombination accordingly. The corrected curves are shown in Fig. 2. After complete photolysis at pH 7.2 recombination is autocatalytic, because the flash is followed by conversion from the R to the T structure within the dead time of the instrument, and recombination is accompanied by reconversion to the R structure, which accelerates the reaction. After partial photolysis initial recombination is much faster, which indicates that Hb(CO)₃ remains in the R structure (Fig. 2a). In human haemoglobin the overall kinetic behaviour is similar to this at both pH 7.2 and 6.0, with and without organic phosphates.

In *Xenopus* haemoglobin at pH 6.0 with inositol hexaphosphate (IHP) full photodissociation produced a time course that no longer accelerated, indicating that the tetramers of *Xenopus* haemoglobin remained in the T structure as they took up CO. On partial photolysis the time course of CO recombination was no faster than on complete photolysis. This is the decisive result, because it suggests the absence of a significant fraction of Hb(CO)₃ tetramers in the R structure, and is characteristic of haemoglobins exhibiting the Root effect (Fig. 2b).

The discovery of dissociation into dimers in the kinetic experiments raised the question of whether this could have contributed to the absence of cooperative oxygen binding in our equilibrium measurements at pH 6. However, the haemoglobin concentrations used for these were 9–15 times higher than for the kinetic measurements, and the fraction of dimers present was no more than 5%.

Stereochemical interpretation of the Root effect

In the T structure of all haemoglobins that exhibit a Bohr effect, the imidazole of the C-terminal histidine HC3 β forms a salt bridge with Asp or Glu FG1 or Glu F6. Formation of that salt bridge raises the pK of the histidine such that protons are bound. The C-terminal carboxyl of the same histidine forms a salt bridge with the invariant Lys C5 α in the T structure of all haemoglobins, regardless of whether they show a Bohr effect or not³. Previous electron density maps of the R structure have shown no density for His HC3 β , whence it was believed to be waving about freely, but a recent high resolution map of human oxyhaemoglobin at -2 °C shows that it is in fact anchored, and that its density may previously have been smeared out by its high temperature factor (B. Shaanan, personal communication). The new map shows that its carboxyl group forms a salt bridge with Lys HC1 of the same β chain and that its imidazole is free; the distance between its N ϵ and the carboxyl of Asp FG1 has increased from 2.8 Å in the T structure to 9 Å in the R structure. Lys HC1 β , to which the terminal carboxyl binds in the R structure, is invariant in mammals, but in those fish haemoglobins that exhibit a Root effect it is replaced by glutamine, and in *Xenopus* by glycine, neither of which would bind the carboxyl (Figs 3 and 4; Table 1). Thus less energy is needed to move His HC3 β towards Asp FG1 β on going from the R to the T structure.

In mammals, position F9 β is occupied by cysteine whose sulphhydryl group takes up one of two alternative positions. In one of them it is tucked away in the pocket between helices F and H, in close contact with the side chain of Tyr HC2 with which it shares the pocket; in the other it is external. In the R structure the SH group oscillates between the two positions, and the equilibrium varies with the spin state of the haem. Low spin favours the internal, high spin the external position (Fig. 3b)¹². In the T structure the sulphhydryl group is out of the tyrosine pocket, but now it is screened from access to reagents by the C-terminal histidine HC3 β ; it is in contact with that oxygen of the C-terminus that is not bonded to Lys C5 α (Fig.

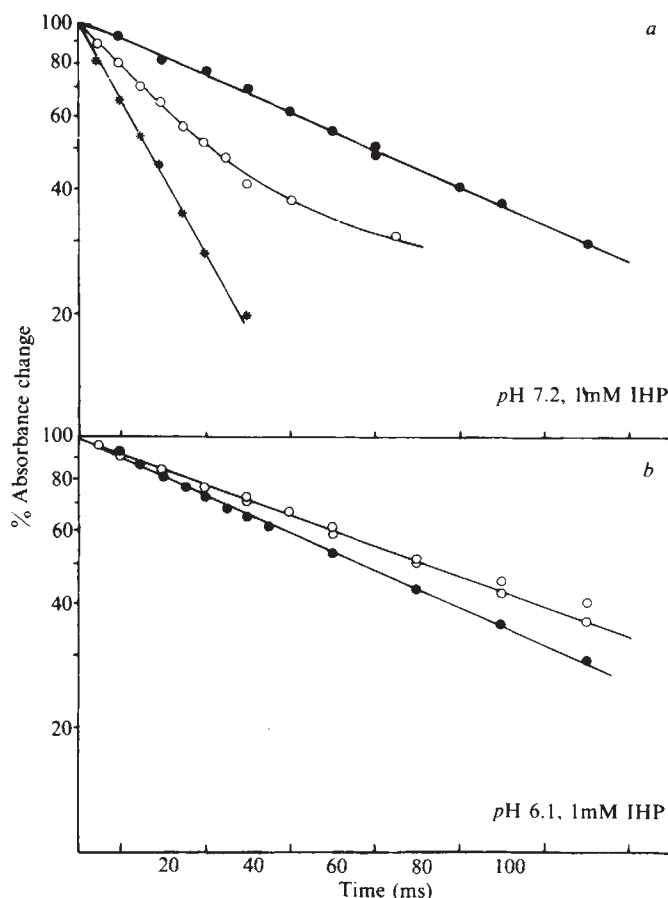


Fig. 2 Recombination of *Xenopus* haemoglobin with CO at 20 °C. The time course of the reaction was followed at 436 nm, using a flash photolysis apparatus similar to that described previously^{5,6}. The intensity of the flash for the partial photolysis experiments was reduced by interposing appropriate neutral density filters. Haemoglobin (concentration = 20 μ M haem) in 0.1 M bis-Tris buffer + 0.1 M NaCl + 1 mM IHP; total CO 100 μ M. *Xenopus* haemoglobin is partially dissociated into dimers in the ligated form, and the dissociation is pH dependent. At 5 μ M haemoglobin in 0.1 M bis-Tris, the fraction of dimeric HbCO is 5% at pH 7.2, 15% at pH 6.7 and 40% at pH 6.2. The fraction of dimers was measured as a function of concentration and the curves shown were corrected for the appropriate fraction by extrapolation to infinite concentration. ●, 100% photodissociation; ○, 11% photodissociation; *, 5% photodissociation.

3a). No matter where it is, the sulphhydryl forms no, or only very weak, hydrogen bonds with its neighbours; no such bonds have been observed in crystal structures of small organic compounds; when on SH approaches an oxygen, the H–O distance is the same as the sum of the van der Waals radii—2.4 Å (ref. 13). What happens when the cysteine in haemoglobin is replaced by serine? On the basis of their partition coefficients between octanol and water, SH was found to be 25 times more hydrophobic than a hydroxyl group¹⁴. Judged by their effects on the vapour pressure of cysteine and serine in water, the hydration potential of a hydroxyl group was found to be more favourable than that of a sulphhydryl group by 3.8 kcal mol⁻¹ (ref. 15). Consequently the side chain of serine is likely to prefer the external, exposed position to the hydrophobic internal one between helices F and H. In the T structure, the OH of serine F9 is so placed that it can donate a hydrogen bond to the unbound terminal oxygen atom of His HC3 β and accept a hydrogen bond from the peptide NH of His HC3 β (Figs 3c and 4). These bonds stabilize the C-terminal salt bridges in the T structure. They would therefore raise the allosteric constant *L*, lower the oxygen binding constant *K_T* and raise the pK of His HC3 β . At low pH the hydrogen bonds may also allow His

HC3 β to form a salt bridge with Glu FG1 β in the R structure. If it did, acid pH would lower not only K_T but also K_R . We suggest that the hydrogen bonds formed by Ser F9 β are the prime cause of the Root effect. This hypothesis led Perutz to predict that the Root effect should be absent in fish haemoglobins from which His HC3 β has been enzymatically cleaved. Goss, Parkhurst and Perutz have tested this prediction in carp haemoglobin and found that such cleavage does indeed cause the Root effect to be inhibited and the Bohr effect to be halved (unpublished data).

Variations of the Bohr effect in different species

In human haemoglobin that part of the alkaline Bohr effect that is not due to His HC3 β was found to be linked to anion binding¹⁶. In stripped haemoglobin in 0.1 M chloride it arose from the binding of chloride ions to Val 1 α and Lys EF6 β ¹⁷. In carp haemoglobin that same part is much larger even in deionized solution (~1.8 protons per tetramer, compared to 1.2 in human)^{16,18}. Where does it come from? The amino acid sequence shows that Ser 1 α is acetylated, so that it can contribute no Bohr protons. Val 1 β is free but has never been found to contribute to the Bohr effect of other haemoglobins. Apart from the haem-linked histidines which contribute no Bohr protons and the C-terminal His HC3 β whose contribution we have just discussed, the only histidine present is FG4 β . This is far removed from any other ionizable group, is known not to contribute significantly to the alkaline Bohr effect in human haemoglobin¹⁷ and is present in tadpole haemoglobin which has only a reverse Bohr effect. The structure shows no paired, buried carboxylates which would have pK values in the physiological range. The evidence suggests that the high concentration of cationic groups between the two β chains causes the pair of Lys EF6 β to act as alkaline Bohr groups, as they were found to do in human haemoglobins¹⁷. The position of Lys EF6 is shown in Fig. 5.

In human haemoglobin in 0.1 M chloride the alkaline Bohr effect vanishes at pH 6 and is reversed below that pH. This is known as the acid or reverse Bohr effect. About half of it is contributed by His H21 β ; the origin of the other half is unknown¹⁷. In fish where residue H21 β is Arg, the acid Bohr effect is absent.

Xenopus haemoglobin does not exhibit as strong a Root or Bohr effect as do carp and trout IV haemoglobins. For instance, we found that its azide methaemoglobin does not show the

striking transition to higher spin at pH 6 exhibited by the two fish haemoglobins¹⁹. *Xenopus* haemoglobin has conserved the essential Ser F9 β , Glu FG1 β and His HC3 β , and it has Gly rather than Lys in position HC1 β to prevent anchoring of the C-terminus in the R structure (Table 1). On the other hand, it has a Lys at F6 β , one turn of π -helix removed from Glu FG1 β and capable of competing with His HC3 β for the essential salt bridge with that Glu (Fig. 4). This competition may weaken its Root and Bohr effects.

Other amphibian haemoglobins show much weaker alkaline Bohr effects or even none at all. The major haemoglobin component of the large American bullfrog *Rana catesbeiana*, when stripped and in dilute solution, shows a Bohr effect of $\Delta p_{50}/\Delta pH = -0.16$, compared with -0.56 in *Xenopus*²⁰. Position F9 β is occupied by Ser, as in *Xenopus*, but its influence is counteracted by the substitution Gly for Glu in position FG1 β . In consequence His HC3 β cannot have its pK raised in the T structure by the usual strong salt bridge with Glu FG1 β , but can only interact more weakly with Glu F6 β which is one turn of π -helix away from residue FG1 β (Table 1, Fig. 4). His H21 β , which contributes to the acid Bohr effect in human haemoglobin A, is replaced by Lys. Tadpole haemoglobin of *R. catesbeiana* in the absence of phosphates has only a reverse Bohr effect²¹. Here Ser F9 β is replaced by Ala, Glu FG1 by Asn, and Ser 1 α is acetylated, so that the alkaline Bohr effect is inhibited entirely. On the other hand, His H21 β is present, consistent with its role in the acid Bohr effect of human haemoglobin. The two major fractions of the adult haemoglobin of the European frog *Rana. esculanta* exhibit only a weak alkaline Bohr effect²². The amino groups of Gly 1 α are free to contribute to it. The sequence of the major component of the β chain is similar to that of *R. catesbeiana*, but position FG1 β is occupied by Asn, so that the strong salt bridge with His HC3 β is inhibited as in *R. catesbeiana*. These are the only amphibian β sequences known.

Haemoglobin of the shark *Heterodontus portusjacksoni*, a cartilaginous fish without a swim bladder, exhibits a weak alkaline Bohr effect²³. Position F9 β is occupied by Ala; residue FG1 β is Glu, which could produce a normal alkaline Bohr effect by forming a strong salt bridge with His HC3 β if it were not for a Lys in position F6 β , one turn of π -helix away, competing with His HC3 β for that salt bridge. The lungfish *Lepidosiren paradoxa* also lacks a swim bladder; its haemoglobin exhibits no Root effect and has an alkaline Bohr effect similar in magnitude to that of human haemoglobin²⁴. It does have Ser at F9 β and Glu at FG1 β , but as in *Xenopus* and in the shark its salt bridge with His HC3 β is weakened by competi-

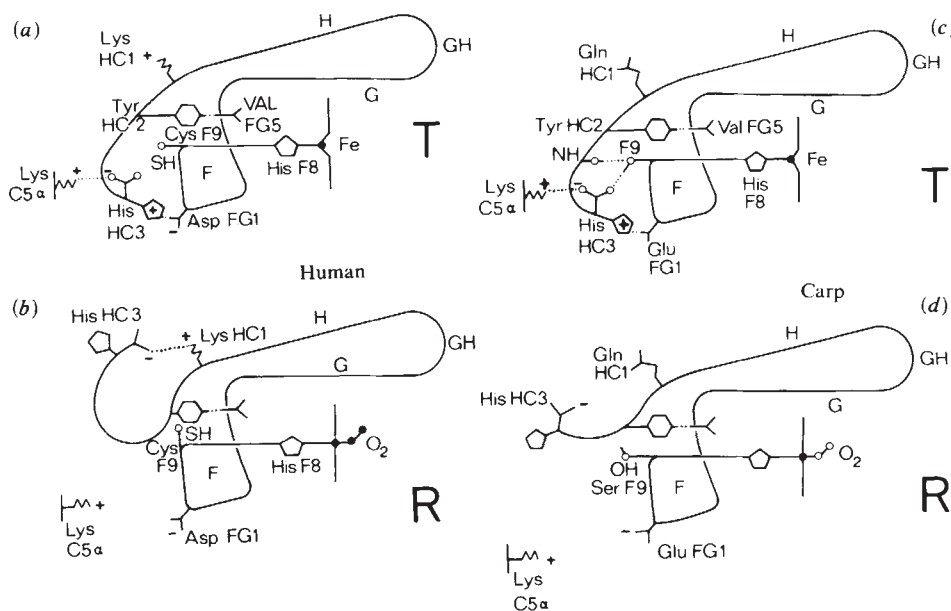
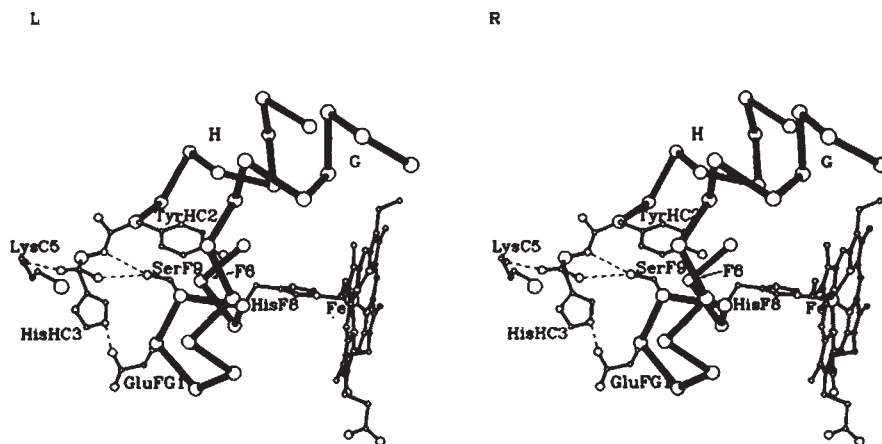


Fig. 3 C-terminal salt bridges and the role of residue F9 β in the T and R structures of mammalian and fish haemoglobins. *a*, Human T structure; *b*, human R structure; *c*, carp T structure; *d*, carp R structure. The letters F, G and H denote helical segments; FG, GH and HC denote non-helical segments. The same notation is used in Figs 4 and 5.

Fig. 4 Stereodiagram showing the bonds at the C-terminus of the β chain in the T structure of teleost fish haemoglobin. The amino group of Lys C5 α donates a hydrogen bond to one of the carboxylate oxygens of His HC3 β , and the OH of Ser F9 β donates a hydrogen bond to the other oxygen. The imidazole donates a hydrogen bond to the carboxylate of Glu FG1. The main chain NH of His HC3 donates a hydrogen bond to the lone pair electrons of the OH of Ser F9. Note the direct link from the haem iron via His F8 and Ser F9 to the C-terminus, which would therefore feel the movement of the iron towards the porphyrin that occurs on oxygen binding.



tion from residue F6 β , in this instance a His. Lungfish haemoglobin has an additional residue, a Met, at the amino end of the α chain. It is not clear if this could contribute to the alkaline Bohr effect by binding a chloride ion jointly with Arg HC3 of the opposite α chain, as Val 1 α does in mammals.

Rat haemoglobin

IHP causes the Hill's constant of three of the major components of rat haemoglobin to approach unity at pH 6²⁵. The only substitutions of polar residues that distinguish rat from human haemoglobin lie on the surface and are not of a kind that would affect the stability of the T structure in the absence of IHP. On the other hand, the dominant fraction III β has a net charge higher by +5 than the human β chain, so that it may bind IHP so strongly as to produce a Root effect. These observations show that a Root effect may be produced by an alternative mechanism to that operating in fish.

The $\alpha_1\beta_2$ contact

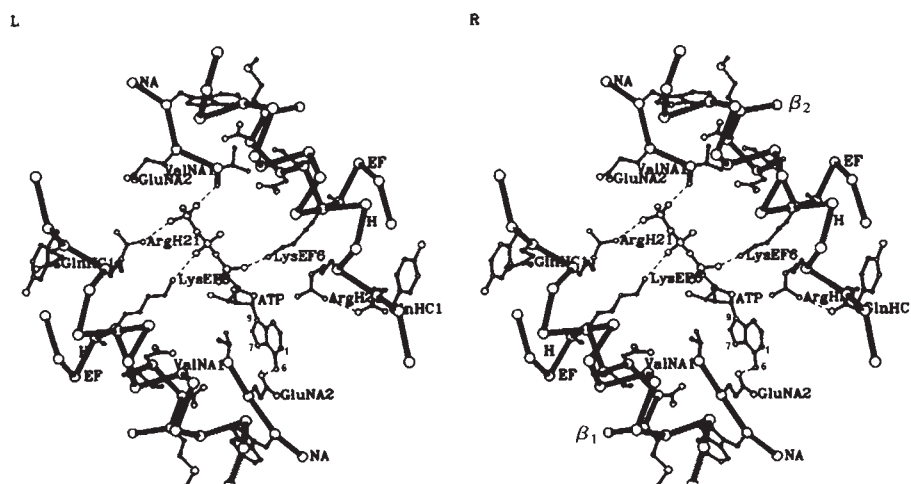
The $\alpha_1\beta_2$ contact works as a snap action switch between the two alternative quaternary structures: in the T structure of all vertebrates a groove formed by Val FG5 β_2 is filled by the side chain of residue C6 α_1 , while in the R structure that same groove is occupied by the side chain of residue C3 α_1 . In mammals C3 α_1 is threonine whose side chain merely interacts with water molecules. In fish C3 α_1 is glutamine whose amide group may either interact with water or form weak hydrogen bonds with Asp G3 β_2 in the T structure and Asp G1 β_2 in the R structure.

In *Xenopus* C3 α_1 is lysine. The atomic models suggest that its amino group would form a salt bridge with Asp G3 β_2 in the T structure, and with Asp G1 β_2 in the R structure. Apart from these differences, the R $\rightarrow$ T switch mechanism is as in human haemoglobin (for illustrations see ref. 26).

ATP and GTP binding sites

Mammals and frogs use D-2,3-diphosphoglycerate (DPG) as an allosteric effector, but teleost fish use ATP, GTP or inositol pentaphosphate^{27,28}. While in mammals DPG lowers the oxygen affinity more than ATP, the opposite holds in teleost fish²⁹, which suggests that the stereochemistry of the binding site must be different. There are three clues to its nature. Carbamylation of Val NA1 β inhibits ATP binding³⁰; mammalian haemoglobins that use DPG as a regulator have a hydrogen donor side chain in position NA2 β (His, Gln or Asn) and they have His in position H21 β . Teleost fish have either Glu or Asp in position NA2 β , and Arg at H21 β . We found that substitution of those side chains in the atomic model of human deoxyhaemoglobin produces a constellation of charged groups stereochemically complementary to strain-free models of ATP or GTP. When ATP is bound, the carboxylate of either Glu or Asp NA2 β_1 accepts a hydrogen bond from the N-6 amino group of adenine; the amino group of Val NA 1 β_2 and the guanidinium group of Arg H21 β_1 each donate a hydrogen bond to (PO₄)⁻, while Lys EF6 β_1 and β_2 donate hydrogen bonds to either of the other two phosphates, thus neutralizing the four

Fig. 5 Suggested ATP binding site of fish haemoglobins. N-6 of the adenine donates a hydrogen bond to the carboxylate of Glu NA2 whose side chain is oriented so that $\chi_1 = 60^\circ$. The purine is in the *anti* conformation with respect to the ribose. The ribose is C3' *endo* and C5'-O5' is *trans*. If ATP is replaced by GTP with the proton bound to N-7, which is unusual, then the purine can remain in the *anti* conformation and N-7 can donate a hydrogen bond to Glu NA2. If the proton is in the usual position on N-1, no hydrogen bond is possible in this conformation. A good hydrogen bond can be made by turning the purine by 180° about the N-9-C-1' bond to the *syn* conformation, and the side chain of Glu NA2 about the C α -C β bond to $\chi_1 = -60^\circ$. All the hydrogen bonds described here lie in the plane of the purine ring. In human haemoglobin 2,3-diphosphoglycerate (DPG) binds to the same site as shown here, but forming salt bridges with Val NA1, His NA2, Lys EF6 and His H21. Diagrams of the DPG binding site appear in refs 26 and 57.



negative charges of ATP (Fig. 5). GTP may bind in two alternative conformations. In the normal tautomer of guanine, which has the proton on N-1, the purine would have to take up the *syn* conformation with respect to the ribose so as to allow N-1 to donate a hydrogen bond to Glu or Asp NA2 β . If the proton were to be transferred to N-7, forming the other tautomer, then the purine could be in the *anti* conformation, as in Fig. 5, and N-7 could donate a hydrogen bond to Glu NA2 β . This structure is less likely, because N₇ has never been observed to carry a proton in uncharged guanine. It is intriguing that substitutions at no more than two positions, NA2 and H21, are needed to convert the DPG to an ATP or GTP binding site. In fact, the substitution of Arg for His at H21 may suffice. Lungfish use ATP as an allosteric effector and yet have His at H21. Model building shows that this could accept a hydrogen bond from the adenine NH₂ or donate one to the guanine carbonyl group.

Biological implications

Many fish use the Root effect to secrete oxygen into both the swim bladder and the eye³¹⁻³⁴. Each organ possesses an elaborate vascular network, the rete, which converts a gradient of increasing lactic acid concentration into a gradient of decreasing oxygen saturation by countercurrent circulation³⁵. Frogs do not possess these organs. Why then does *Xenopus* haemoglobin exhibit a Root effect? It is an aquatic frog, and part of its respiratory exchange takes place through capillaries in its skin. Yet this can hardly be the explanation because the same is true for the mainly terrestrial frog *R. esculanta* when it is under water. In both animals about two-thirds of the capillaries for gas exchange are in the lungs and one third is in the skin³⁶; oxygen is taken up mainly through the lungs, while CO₂ is excreted mainly through the skin³⁷. We do not know what, if any, selective advantage the Root effect confers on *Xenopus*, but we have come across one habit unique to *Xenopus* that might give a clue. *Xenopus* lives in muddy pools in Southern Africa. E. M. Deuchar writes: "When some of the pools dry up in summer, *Xenopus* has to burrow into the mud to avoid desiccation, and here it remains quiescent, aestivating, until the next rainy season"⁵⁸. Under these conditions the frog would be short of oxygen. Conceivably the Root effect would then allow a greater fraction of the oxygen stored in the blood to be used by the tissues.

The constellation of polar residues needed to produce the Root effect and the accompanying large Bohr effect in teleost fish seems to consist of Lys EF6, Ser F9, Glu FG1, Arg H21 and His HC3, all on the β chains. We have not found any residues in the α chain that contribute directly, though some may do so indirectly, by biasing the allosteric equilibrium towards the T structure. In many amphibian and fish haemoglobins most or even all these essential polar residues have been conserved even when they lack a Root effect and exhibit only weak Bohr effects. In some instances this happens because their influence is countered by a substitution elsewhere: in *Xenopus* and shark by Val F6 β $\rightarrow$ Lys, and in the lungfish by Val F6 β $\rightarrow$ His, which compete with the C-terminal histidine for the salt bridge with Glu FG1 β . In the adult haemoglobins of the two terrestrial frogs a strong alkaline Bohr effect is inhibited by the substitutions Glu FG1 β $\rightarrow$ Gly or Asn and a weaker one is generated by the substitution Val F6 β $\rightarrow$ Glu. In the tadpole of *R. catesbeiana* the substitutions Ser F9 β $\rightarrow$ Ala and Glu FG1 β $\rightarrow$ Asn seem to abolish the alkaline Bohr effect, even though Glu still occupies the position F6 β ; the substitution Lys H21 β $\rightarrow$ His, and possibly others still unknown, introduce a reverse Bohr effect. Trout I haemoglobin shows no response to any heterotropic ligands even though it exhibits normal haem haem interaction, because all the polar residues involved in both the alkaline and acid Bohr effects and in phosphate binding are replaced by neutral ones: Lys EF6 β is replaced by Leu, Ser F9 β by Ala, Glu FG1 β by Asn, Arg H21 β by Ser and His HC3 β by Phe^{3,38}. Trout I haemoglobin provides the most compelling evidence that heterotropic ligands exert their influence at a small number of specific sites. One cannot help being struck by the beautiful design of the stereochemical mechanism that gives rise to the strong Root and Bohr effects in teleost fish, and by the haphazard way that evolution has chosen to weaken, inhibit and even reverse these effects in a variety of different ways when they are not needed. It is an example of what Jacob calls "evolutionary tinkering"³⁹.

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LETTERS TO NATURE

Neutron oscillation as a source of excess sub-GeV antiprotons in galactic cosmic rays

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The recent detection^{1,2} of an antiproton/proton ($\bar{p}/p$) ratio at energies much less than 1 GeV is several orders of magnitude above that predicted for $\bar{p}$ production from primary cosmic ray collisions. It is well recognized that there is no mechanism to explain this sub-GeV (130–320 MeV) $\bar{p}$ excess although many suggestions have been made. For example, Eichler³ considers secondary production in high-energy collisions while Kiraly⁴ *et al.* suggest black hole evaporation as a $\bar{p}$ source. Most of these suggestions have inherent difficulties such as the imposition of severe constraints on the sources to suppress excessive γ -ray production. We postulate here that the phenomenon of neutron–antineutron ($n\bar{n}$) oscillations as a signature of grand unified theories may operate in neutron-rich astrophysical sources such as supernovae to produce the required low-energy galactic antiproton background.

Theoretical predictions of the generation of antiprotons in cosmic rays as a result of collisions of primary protons at relativistic energies would require $\bar{p}/p$ to decrease with decreasing energy, as the threshold for production of $\bar{p}$ from these mechanisms is ~ 2 GeV. This is in contrast with the observations where $\bar{p}/p$ increases at lower energies, the ratio being $>10^{-4}$ at 130–320 MeV. The observation of Buffington *et al.*^{1,2} would imply a $\bar{p}$ energy density of $\sim 10^{-4}$ eV cm⁻³ inside our Galaxy. In some hypotheses the $\bar{p}$ s have been considered to be primary cosmic rays from antigalaxies⁵. But the fact that the reported He/He ratio is significantly lower than the $\bar{p}/p$ ratio would raise difficulties with such models. A recent trend has been to assume the $\bar{p}$ s as secondaries and propose scenarios for decelerating them to sub-GeV energies. For example, $\bar{p}$ s could be produced at a large cosmological redshift and decelerated by the expansion of the Universe. Unfortunately this would imply an energy density in high-energy neutrinos (simultaneously produced in the high-energy collisions) well in excess of the Reines⁶ experimental limit on cosmic neutrino background at $E > 0.5$ GeV. Other models of deceleration of $\bar{p}$ s within the Galaxy also have undesirable features such as excessive γ -ray production. This would require $\bar{p}$ s to be produced only in compact optically thick sources that would reduce the γ -ray flux. Moreover there are constraints on the density of the traversed matter. Clearly a mechanism is needed to account for the excess production of sub-GeV $\bar{p}$ s at the same time without producing γ rays and high-energy neutrinos. Preferably one should have a means of producing the $\bar{p}$ s directly at sub-GeV energies without having to invoke deceleration mechanisms. Obviously, the direct low-energy production of $\bar{p}$ s would violate baryon number conservation. Fortunately recent progress in grand unified theories⁷ has provided for just such a situation. The phenomenon of $n\bar{n}$ oscillation has been suggested as a consequence of grand unification^{8,9}. It has also been invoked to generate the cosmic-ray antiproton flux by the spallation of cosmic-ray nuclei in interstellar space which is, however, low because of the low $^4\text{He}/p$ ratio¹⁰.

The $n\bar{n}$ mechanism is expected to be important wherever large fluxes of low-energy neutrons are produced or are present. A supernova explosion, for instance, is expected to release a very large number of neutrons. There are several ways in which the neutrons may be generated, for instance the supernova shell

is sufficiently dense for the heavy nuclei to interact and produce neutrons by breakup or charge exchange. Nuclear statistical equilibrium distributions dominated by extremely neutron-rich nuclei are expected for the large neutron-to-proton ratio characterizing the supernova material. One expects¹¹ that the total ratios of neutrons to protons will reach 2–8. In effect, every supernova explosion might shoot out 10^{57} free neutrons into interstellar space, the rest having been captured. We will now briefly discuss the phenomenon of $n\bar{n}$ oscillations. This transition is a first-order process involving a baryon number change of 2. This is analogous to the well known phenomenon of strangeness oscillation in the neutral K-meson system, that is $K_0 \leftrightarrow \bar{K}_0$, which involves a strangeness change of 2. Several theoretical models have been proposed to estimate the average transition time for the oscillations $n\bar{n}$. The characteristic time of such oscillations is predicted to be between 10^5 and 10^7 s and the associated transition energies ($\Delta\rho$) are of the order of 10^{-20} eV (refs 8, 9). The β decay of the antineutron so formed results in an antiproton. So the net result is the transformation of a neutron into an antiproton and this can take place at low energies. Therefore, this may be an appropriate phenomenon for the production of low-energy $\bar{p}$ s without any of the attendant γ rays which is the weakness of other mechanisms. In the presence of the magnetic field, the $n\bar{n}$ oscillations are inhibited because of magnetic splitting of the neutron energy levels, $\Delta E = g\mu B$, where g is the anomalous gyromagnetic ratio of the neutron, $\mu = (e\hbar/2m_n c)$ and B is the magnetic field strength. Writing the hamiltonian in the presence of the magnetic field, the usual Schrödinger perturbation theory then gives for the transition rate $\bar{n}/n$ as¹⁰

$$\frac{\bar{n}}{n} \approx \frac{1}{2} \left[\frac{\Delta\rho}{\Delta E} \right]^2 \quad (1)$$

where $\Delta\rho = \hbar/\tau \rightarrow 10^{-32}$ erg, τ being the oscillation time of 10^5 s and $\Delta E = 9 \times 10^{-24} B$ erg, where B is in Gs. As $\bar{n}$ subsequently decays to antiprotons, equation (1) also gives the ratio $\bar{p}/n$. As mentioned above, every supernova explosion shoots out 10^{57} free neutrons into interstellar space where the average magnetic field is known to lie between 10^{-6} and 10^{-7} G. With this value of B in equation (1) then gives for $\bar{p}/n$,

$$\frac{\bar{n}}{n} = \frac{\bar{p}}{n} = \left[\frac{10^{-32}}{9 \times 10^{-24}} \right]^2 \frac{1}{(10^{-7})^2} \approx 10^{-4} \quad (2)$$

As observed above $n/p \sim 10$ for supernova matter, thereby giving $\bar{p}/p \sim 10^{-3}$. This would imply that out of the 10^{57} free neutrons shot out of the supernova into the interstellar space, 10^{54} of them would get converted into low energy antiprotons. A cosmic-ray proton density of 1 eV cm⁻³ and a galactic volume of 10^{68} cm³ would give $\sim 10^{59}$ protons. The observed $\bar{p}/p$ ratio would then require 10^{55} antiprotons to be present in the Galaxy (an observed energy density of 10^{-4} eV cm⁻³). We thus see that a few supernova explosions are fully capable of accounting for the background antiproton flux.

The value of 10^{-7} G for the interstellar field used in equation (2) is rather low; a commonly quoted value is $\sim 10^{-6}$ G. This is the value given by equipartition arguments though there is no physical basis for it. It could be different from 10^{-6} G, as discussed by Burbidge¹², in the context of synchrotron radiation from extended galactic and extragalactic sources. The use of 10^{-6} G in equation (2), however, reduces $\bar{p}/n$ to 10^{-6} , which would imply (with $n/p \sim 10$) that each supernova explosion would produce only 10^{52} antiprotons. So, the observed $\bar{p}$ background density of 10^{-4} eV cm⁻³ can be produced only after 1,000 supernova explosions. Assuming a rate of one explosion in 10 yr (ref. 13) would require time periods $\sim 10^5$ yr (about the same order as that required for the particles to spread over the Galaxy) for the observed antiproton background to build

up. We can thus trade off a higher value for the magnetic field with more explosions to build up the antiproton background. Thus interstellar fields of a few microgauss can over a period of a few hundred thousand years give rise to an antiproton background of about the observed galactic density.

The effect of solar activity on the background cosmic ray intensity is interesting. It is well known that during flares, the solar wind sweeps away the low energy cosmic rays, resulting in Forbush decrease. On the other hand, the generation of antiprotons through $n\bar{n}$ oscillations occurs additionally during solar flares at which time a large neutron flux is produced¹⁴.

We conclude that the phenomenon of $n\bar{n}$ oscillation occurring in the aftermath of a large neutron release in supernova explosions can account for the observed sub-GeV antiproton flux in cosmic rays. We note that this mechanism does not suffer from the attendant production of large fluxes of unaccountable γ rays or high-energy neutrinos like other scenarios, nor is there any need for deceleration mechanisms, as the antiprotons are produced at MeV energies straightaway.

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Intense $\text{Ly}\alpha$ emission from Uranus

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The existence of intense atomic hydrogen $\text{Ly}\alpha$ emission from Uranus is demonstrated here by utilizing the monochromatic imaging capabilities of the International Ultraviolet Explorer (IUE) spectrograph. Our observations show increased emission in the vicinity of Uranus superimposed on the geocoronal/interplanetary background. If resonant scattering of solar $\text{Ly}\alpha$ is the source of the 1.6 ± 0.4 kR disk averaged brightness, then very high column densities of atomic H above the absorbing methane are required. Precipitation of trapped charged particles—aurora—could explain the emissions. This would imply a planetary magnetic field.

The $\text{Ly}\alpha$ line of atomic hydrogen is often the most easily detected UV emission from the upper atmosphere of a planet; in the case of the major planets with largely H_2 atmospheres, the line may be quite bright. The resonant scattering of the intense solar radiation provides a means of estimating the upper atmosphere H column density, which in turn depends on the eddy diffusion transport rate. If a planetary magnetic field is present, precipitating charged particles can produce intense emissions particularly near the polar latitudes.

Darius and Fricke¹ recently described a measurement of the $\text{Ly}\alpha$ brightness of Uranus with the IUE spacecraft; the value was surprisingly high, 1.9 kR. However, two questions caused uncertainty. First, because of the imperfect image quality of the telescope, it was not certain how much of the 3.8 arcs planetary disk passed through the nominal 3 arcs diameter small aperture. Second, the ratio of the solid angles of the large and small apertures—used to subtract the dominant terrestrial airglow from the total signal—was uncertain. A new and more reliable determination of the $\text{Ly}\alpha$ brightness of Uranus is now

described that uses the IUE spacecraft but with a different technique. In particular, the imaging property of the UV instrumentation was utilized. When the planetary image is placed in the large aperture, an ~ 9 arc s monochromatic image is produced in the focal plane of the low resolution spectrograph at the wavelength of $\text{Ly}\alpha$; the planetary $\text{Ly}\alpha$ emission appears as a feature on top of the larger terrestrial/interplanetary feature which covers an area in the focal plane equivalent to $\sim 16 \times 27$ arc s. As a result, the strength of the planetary emission relative to the geocoronal/interplanetary contribution can be assessed directly and $\text{Ly}\alpha$ emission from Uranus can be demonstrated beyond doubt. In addition, to reduce the geocoronal contribution, the observation was performed on 3 March 1982 when the local spacecraft zenith angle of Uranus was near minimum at the time of orbit apogee. Also a determination² of the variation of the $\text{Ly}\alpha$ emission with IUE orbit parameters enabled us to scale with confidence $\text{Ly}\alpha$ background measurements for the large aperture obtained at other times in the shift so that the terrestrial/interplanetary contribution could be subtracted.

Two 2-h exposures of Uranus (SWP 16468 and SWP 16469) were obtained beginning at 11 h 58 min and 14 h 26 min UT. The planetary disk was 3.7 arc s. This was followed by three exposures of the terrestrial airglow (SWP 16470, SWP 16471 and SWP 16472) 330 arc s south-west of the planet. The exposures began at 16 h 56 min UT and ended at 20 h 51 min UT; their total integrated observing time was 185 min. The line by line spectra—in which a datapoint corresponds to an area 1.1 arc s in the dispersion direction by 2.1 arc s high—were used to obtain a two-dimensional plot of the focal plane flux. Figure 1 shows a projection of the resulting data set at $\text{Ly}\alpha$. Figure 1a shows the sum of the three airglow only exposures whereas Fig. 1b also contains the contribution of Uranus. There seems to be no question that Uranus is a strong emitter, producing a localized hump on top of the flat topped distribution of ~ 1.2 kR of terrestrial/interplanetary airglow. Finally, Fig. 1c shows our best estimate of the Uranus only emission obtained by subtracting Fig. 1a from Fig. 1b and multiplying by a factor of 3. (Note that Fig. 1a has both been corrected for differences in observing times and multiplied by 0.67 to correct for the variation in geocorona with time.) Summing the flux over a circle of 10 arc s diameter and using the standard IUE calibration of Bohlin *et al.*³ implies a uranian disk averaged brightness of 1.6 ± 0.4 kR. The value of the brightness is dependent on measuring the flux in the tail of the distribution. Thus, if one arbitrarily rejects all values outside of a 7 arc s diameter instead of a 10 arc s diameter, the computed value drops to 1.1 kR. Hence, the imaging technique used here is important. As a check on the subtraction, the airglow contribution was increased before subtraction. When the value for Uranus dropped to 1.2 kR, most of the data points in the region of airglow only were negative. Similarly, decreasing the airglow contribution led to an upper limit of 2.0 kR. The effect of absorption by interplanetary H is difficult to ascertain because the line width of the emitted radiation is not known. In general it will raise the value above that reported here.

What is the cause of this strong emission? First, consider resonant scattering from an optically very thick layer of atomic H. Assuming a cosine distribution⁴, the implied uranian subsolar brightness is 2.4 kR. This is to be compared with a subsolar brightness for Jupiter of 8.5 kR obtained in January 1982 (ref. 5). Correcting for the different heliocentric distances gives that the reflectivity of Uranus is 3.8 times higher. Using the calculations of Clarke *et al.*⁶ scaled to a solar $\text{Ly}\alpha$ flux at the Earth 3.6×10^{11} photons $\text{cm}^{-2} \text{s}^{-1}$ (G. J. Rottman, personal communication) implies that the uranian atomic hydrogen column density above the absorbing methane is ≥ 10 times thicker than the case of Jupiter. This would be surprising in that the effect of solar photodissociation to produce H from H_2 is down by a factor of 14; removal of the hydrogen by eddy diffusion transport would have to be two orders of magnitude below the case of Jupiter. The methane density in the high atmosphere may

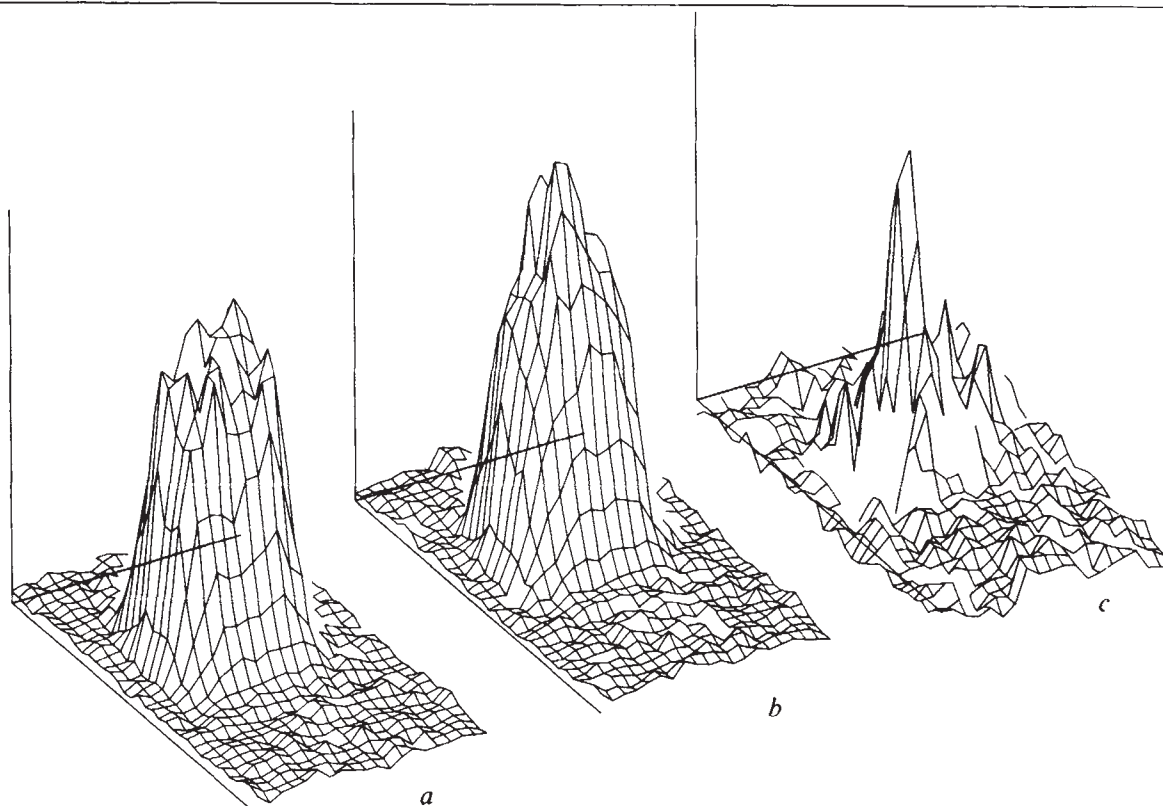


Fig. 1 Projection of the $\text{Ly}\alpha$ flux in the IUE short wavelength spectrograph focal plane showing the $\text{Ly}\alpha$ emission from Uranus. *a*, Sum of three exposures with geocorona only normalized to *b*; *b*, sum of two 2-h exposures with Uranus at the centre of the large entrance aperture; *c*, the uranian $\text{Ly}\alpha$ emission obtained by subtracting the geocoronal contribution from *b* and multiplying the result by 3. The long dimension of the aperture is approximately parallel to the axes pointing upward and to the right. The dispersion direction is approximately parallel to the axes pointing downward and to the right.

be lower than the other major planets⁷ which would increase the depth of the hydrogen column. In any case, the subsolar reflectivity is 60% of that expected from a perfect lambertian surface, a not impossible but quite high value.

An alternative explanation is precipitation of trapped charged particles. Polar aurorae on both Jupiter⁸ and Saturn⁹ have produced $\text{Ly}\alpha$ emissions easily observed by IUE. Uranus had its pole in the ecliptic plane pointing approximately earthwards at the time of the observation. Thus polar aurorae could have contributed substantially to the observed brightness.

At present, we are not able to distinguish between the two sources, although the demands on resonance reradiation of solar $\text{Ly}\alpha$ are so great that it forces a prejudice towards trapped charged particle precipitation. The latter in turn implies a planetary magnetic field. Observational signatures of trapped particle precipitation are temporal variations in the $\text{Ly}\alpha$ brightness and H_2 Lyman/Werner band emission². We plan to extend our observations of Uranus to see if these signatures exist.

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Precambrian endoliths discovered

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The earliest known microborings have now been found in late Precambrian ooids (Upper Riphean¹/Vendian², 570–700 Myr) of the Eleonore Bay Group of eastern Greenland. The ooids were originally carbonaceous and underwent silicification after boring occurred. The finding establishes that the habit of microbial boring evolved before the appearance of skeleton-bearing metazoans in the geological record.

Microbial endoliths have bored skeletal fragments and carbonate rock³ throughout the Phanerozoic eon⁴. Precambrian ooids were surveyed for early endolithic activity because microbial endoliths are commonly associated with modern ooids,⁵ whereas epiliths have a much lower frequency of association with them.⁶ Ooids are hard, spherical structures that accrete and form concentric laminae abiotically, that is, without the involvement of carbonate-precipitating microorganisms such as those responsible for stromatolite formation⁵. For these reasons, Precambrian ooids were considered to be microbiologically 'clean' systems in which only endolith fossils were likely to be found.

Twenty-three fossils were found in the thin sections of oolite from level 16 of the Eleonore Bay Group in sample GGU 145426, which corresponds to the no. MGUH 13 807 of the Geological Museum, Copenhagen². The endolithic microfossils (Fig. 1a–d) are present as fillings of boreholes, and thus appear dark against the lighter background of the silica mineralogy of the ooids. Borings in modern ooids (Fig. 1e, f) often become filled with material darker than the white ooidal carbonate

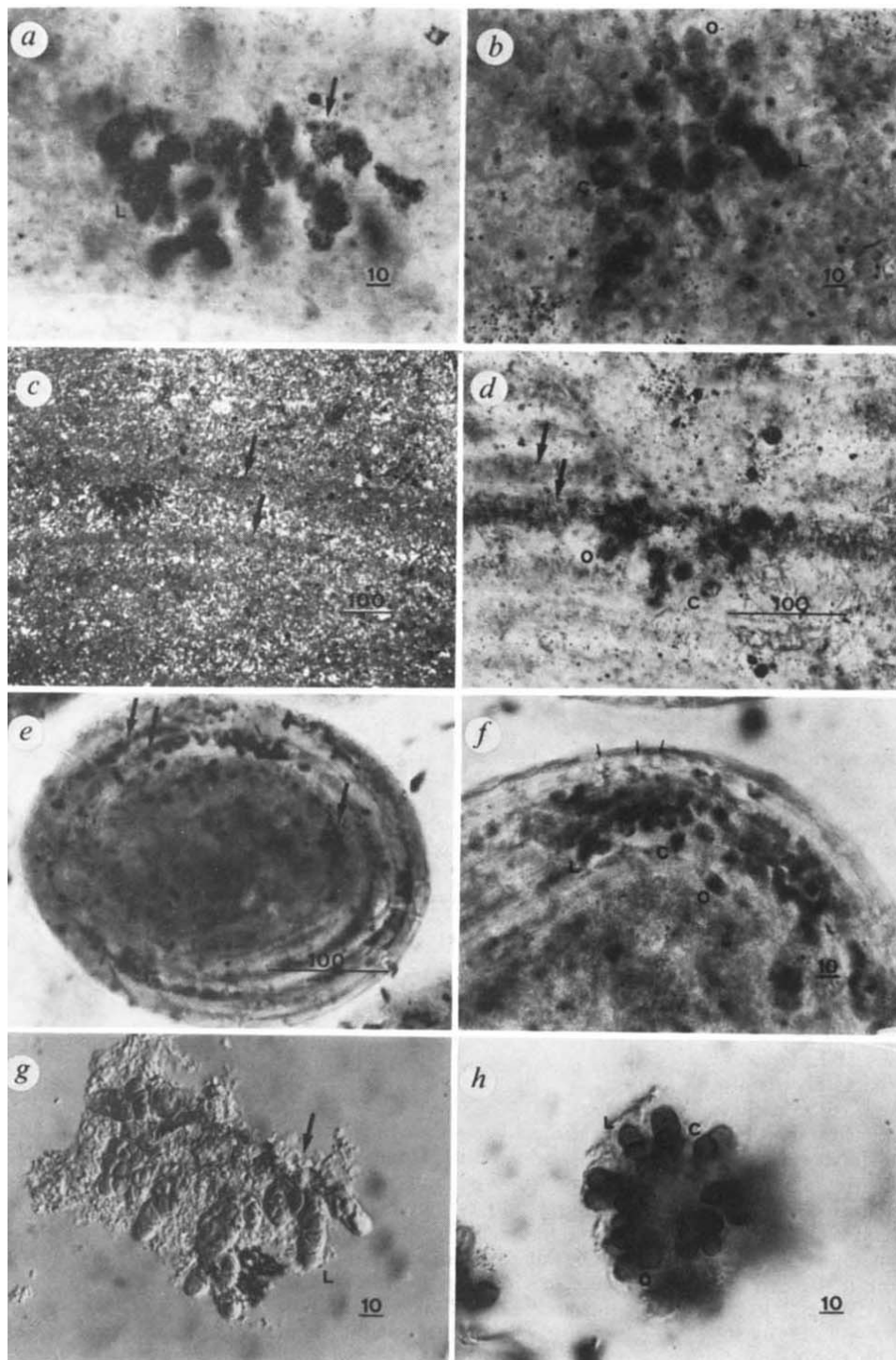


Fig. 1 *a*, Precambrian endolith within a silicified ooid. It is composed of a cluster of short branching filaments (compare with *g*). *L* = longitudinal section of boring. Arrow indicates surface at the time of boring. Scale bar, 10 μ m. *b*, Precambrian endolith cluster transversely sectioned. Individual borings appear in cross (*C*), oblique (*O*) and longitudinal (*L*) section. Scale bar, 10 μ m. *c*, Fossil in *a* shown in mineralogical context; partial cross-polarized light emphasizes concentric laminae (arrows). Scale bar, 100 μ m. *d*, Precambrian endolith aligned along lamina. Laminae (one-time surfaces of the ooid) shown by arrows. Endolith cluster is cut tangentially (off-centre) while ooid is in radial section. Borings are seen in cross-section (*C*) and oblique section (*O*). Scale bar, 100 μ m. *e*, Modern carbonate ooid from Bahamas showing multiple generations of boring along concentric laminae (arrows). Scale bar, 100 μ m. *f*, Empty boreholes are visible at the edge of this modern ooid (small arrows), whereas filled borings are dark against the white ooidal carbonate. Borings in longitudinal section (*L*), cross-section (*C*) and oblique section (*O*). Scale bar, 10 μ m. *g*, Modern decalcified endolithic microorganism *Hyella* (Cyanophyta) in longitudinal view. Arrow indicates the position of substrate surface before the carbonate was dissolved. Scale bar, 10 μ m. *h*, Decalcified *Hyella*, photographed from beneath (optical section), illustrates radiating morphology. Compare with borehole morphology of fossil in *b*. Cells in longitudinal (*L*), cross (*C*) and oblique (*O*) sections. Scale bar, 10 μ m.

(Fig. 1*f*, dark structures), whereas empty boreholes remain clear in transmitted light microscopy (Fig. 1*f*, lacunae in the ooid periphery, small arrows). The fossils (Fig. 1*a-d*) are defined by dark particulate matter of submicrometre size. This material may be finely disseminated pyrite, graphite or bitumen. The sharp outlines of the fossils represent the borehole walls. There are no cellular or extracellular organismal structures present such as those described for other Precambrian microfossils⁷⁻⁹. Thus, the endolithic fossils are actually trace fossils rather than body fossils.

Nearly all the fossils are associated with the concentric laminae of the ooids (Fig. 1*c, d*, arrows). Borings in modern ooids are also preferentially aligned along the laminae (Fig. 1*e*) because the latter represent one-time surfaces of discontinuously growing ooids. Endoliths bore into the ooid after

settling on the surface. Any single ooid may have many laminae containing successive generations of boring (Fig. 1*e*, arrows), or many successive undisturbed laminae containing only isolated borings (Fig. 1*c, d*). The frequency of boring depends on the residence time of individual ooids at the sediment-water interface where boring occurs, or the availability of reproductive propagules of the colonizing endoliths. In the case of the fossil, taphonomic (post-mortem) information loss¹⁰ and diagenetic recrystallization may have removed a portion of the record of endolithic boring.

The morphological features of the fossils are best seen in radial section of the ooid (Fig. 1*a, c*). In this position, the endoliths appear in profile. They are composed of clustered, branching filaments (8–15 μ m diameter), radiating out from the point of entry (at a concentric lamina) in a generally centripetal

direction with respect to the ooid nucleus. The structures are three-dimensional and, as such, cannot be fully represented in a two-dimensional photomicrograph. Drawings made with a camera lucida attachment on the microscope can, however, take three-dimensionality into account by outlining each filament while the microscope is slowly focused up and down. Figure 2 is a camera lucida drawing of the fossil shown in Fig. 1a, c. Filaments branch laterally and dichotomously, and they tend to swell somewhat at their tips. All these are morphological features common to the modern photosynthetic prokaryotic endolith *Hyella* (Cyanophyta, Figs 1g, h).

The ooid in Fig. 1b was cut tangentially during preparation of the thin section. As a result, the cluster of borings was cut transversely. A similar orientation of a cluster of modern *Hyella* is seen in transverse optical section in Fig. 1h. Figure 1d shows a radial section through an ooid. Here, the concentric laminae are visible in their parallel relations (arrows), but the section preparation process ground away all but the tips of the ramifying fossil borings, and they appear both in cross-section (C) and oblique section (O). A similar orientation of borings can be seen in the modern ooid of Fig. 1f. There, small segments of longitudinally sectioned filamentous borings are visible, but most borings have been cut obliquely and in cross-section.

The age of the oldest endoliths has previously been reported to be Cambrian¹¹, and the oldest that were photographically documented were Ordovician¹². S. M. Awramik (personal communication) has found silicified endoliths similar to the modern cyanophyte *Cyanosaccus* in Cambrian rock. The nearly constant association of endoliths with modern ooids is well known⁵ and Harris *et al.*⁶ have compared borings made by several modern endolithic genera with those found in Pleistocene ooids.

The size and morphology of the Precambrian boreholes reported here are most similar to those of the modern endolithic cyanophyte genus *Hyella* (Fig. 1g, h), an organism that bores modern ooids⁶. The only other modern endoliths of comparable size and morphology are the conchosporangial branches of the endolithic stage of the bangiacean rhodophytes (eukaryotes), a group which has been found to have ancient Silurian members¹³. Insufficient evidence exists at present to make closer comparisons between these organisms and the Precambrian fossils. Clearly, however, of all endolithic borehole morphologies known today, only those of photosynthetic endoliths are comparable with the Precambrian ones reported here.

Thus, the earliest known occurrence of microbial endoliths in silicified late Precambrian ooids predates the appearance in the geological record of skeleton-bearing metazoans, whose hard parts were bored from Cambrian time to the present. The morphology of the Precambrian fossils corresponds to that of modern photosynthetic endolithic taxa rather than heterotrophic ones.

The finding of Precambrian microborings in ooids raises a new question for palaeontological evaluations of Precambrian stromatolites, another group of laminated accretional carbonate structures. In his study of modern stromatolites of Shark Bay, Australia, Golubic^{14,15} discussed the importance of endoliths in the destruction of lithified stromatolites. Thus, it must now be recognized that Precambrian stromatolites may have been alternately recolonized by carbonate-precipitating (constructive) microflora, and following lithification events, by carbon-

ate-solubilizing (destructive) ones. This has not been considered in previous interpretations of the Precambrian fossil record. Thus, both past and future reports of silicified microfossils in Precambrian stromatolites need to be evaluated with this distinction in mind.

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Temporal variations in dissolved selenium in a coastal ecosystem

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In oceanic environments biological processes are now known to have a central role in establishing the redox state and spatial distribution of certain dissolved trace elements. Biological effects on the natural cycles of Cr, As, Se, Cd, Ni and Al are inferred from the existence of thermodynamically unfavourable species, from non-conservative vertical profiles or because trace element levels in planktonic debris are such that the subsequent regeneration of material could produce the observed profiles¹⁻⁷. In cases where biological activity has been implicated as a determinant of either redox state or spatial distribution in the oceans no field investigations have yet been made into the relationships between rapid fluctuations of biological parameters and trace element levels and speciation. We now present a temporal study of the response of selenium levels and redox state to pulses of primary productivity in a coastal ecosystem. The sampling time series demonstrated selective assimilation of the lower oxidation state, selenite [Se(IV)]. Ratios of particulate selenium to particulate organic carbon (Se:C) allowed an estimation of the selenium levels in a natural phytoplankton population and the flux of selenium from surface ocean water to deep water.

Selenium is a unique example of a multiple oxidation state trace element in that the thermodynamically unfavourable species, Se(IV), persists throughout the water column in oceanic profiles⁴. This phenomenon may be a result of bioreduction of the dominant higher oxidation state, Se(VI), in surface water and downward transport of the kinetically stable reduced form.

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Fig. 2 Camera lucida drawing of Precambrian endolith in Fig. 1a illustrates its filamentous morphology. The dotted line shows the position of the previous ooid surface. Scale bar, 10 μ m.

The underlying processes which lead to non-conservative behaviour of selenium in the water column can only be investigated in a non-equilibrium context. For this reason we have considered the question of selenium redox chemistry against a background of variations in biological activity in a fjord ecosystem (Bedford Basin, Nova Scotia, Canada) where primary productivity is high and for which a strong biological data base already exists^{8,9}. As this was a pilot study we collected only one sample at 5 m depth at a single station on a weekly basis between 6 January and 28 April 1981. The intentions of this simple scheme were to establish whether (1) such ecosystems produce significant temporal changes in trace element levels; (2) what kind of quantitative data can be obtained from a simple sampling scheme; (3) how further more extensive temporal and spatial sampling schemes should be designed to provide a closed budget for such a system.

Selenium levels in surface waters are typically in the picomolar range. We have therefore applied a recently developed gas-liquid chromatographic (GLC) technique to the accurate resolution of small variations of dissolved selenium species¹⁰. Selenium analyses on the particulate fraction were also carried out by GLC after wet digestion of the particulates retained on a glass fibre filter from 40 l of seawater¹¹. The detection limits and precisions (1σ) at the observed levels are: Se(IV), 10 pmol l⁻¹ ($\pm 4\%$); total Se, 10 pmol l⁻¹ ($\pm 6\%$); Se(VI) (by difference), 10 pmol l⁻¹ ($\pm 10\%$); particulate selenium (PSe) < 0.1 pmol l⁻¹ ($\pm 7\%$).

The sampling period is characterized by four distinct events in the particulate phase (Fig. 1). Nutrient behaviour (Fig. 2a) during the first of these events centred on 14 January is anomalous in that there is a decrease in dissolved $\text{NO}_3 + \text{NO}_2$ but no significant reduction in PO_4 and SiO_2 . The particulate organic carbon (POC) maximum on 21 April is not associated with any rise in chlorophyll levels. These observations suggest that the first and last biomass maxima are not simple algal blooms and they will therefore not be discussed in any detail.

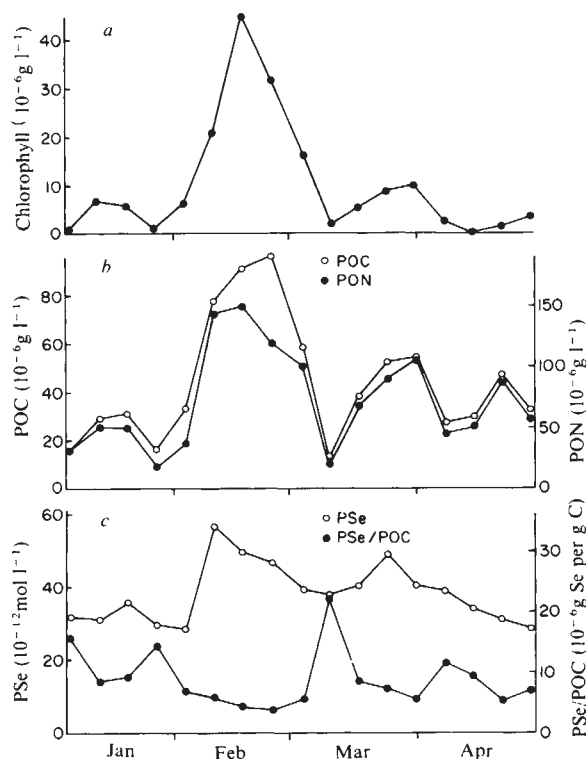


Fig. 1 Temporal changes of biological parameters and selenium in the particulate phase at 5 m depth (Bedford Basin). Samples were taken by Niskin bottle. POC and PON retained on silver filters from 1 l of seawater were quantified using a Perkin Elmer CHN analyser. Chlorophyll was measured by fluorescence spectrometry.

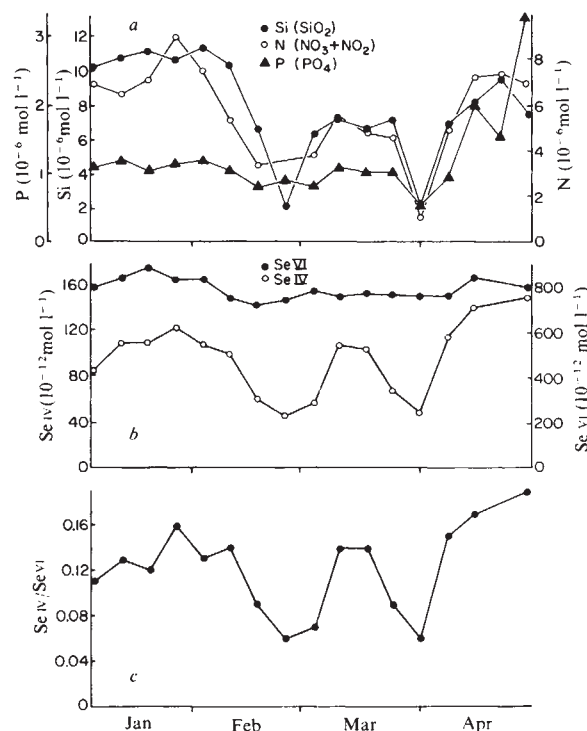


Fig. 2 Temporal changes of nutrients and selenium redox species in the dissolved phase at 5 m (Bedford Basin). Samples were taken by Niskin bottle. Nutrient analyses were carried out on fresh samples using a Technicon autoanalyser.

The second and major event centred between 17 and 24 February is a typical phytoplankton bloom with large increases in POC, particulate organic nitrogen (PON), (PSe) and chlorophyll, and corresponding decreases in SiO_2 , PO_4 , $\text{NO}_3 + \text{NO}_2$, Se(IV) and Se(VI). The decay of this bloom is marked by a rapid regeneration of the dissolved nutrients including Se(IV) (Fig. 2b), with concentrations reaching 65–90% of the pre-bloom levels. Se(VI) does not seem to be regenerated as efficiently as Se(IV) after the bloom.

The third event centred on 31 March is also a characteristic algal bloom but of lower magnitude than the February event. Rises in POC, PON, PSe and chlorophyll levels are mirrored by decreases in the dissolved nutrients and Se(IV) but not Se(VI). The decay of the bloom is again marked by a rapid regeneration of nutrients, including Se(IV) (Fig. 2b), with final concentrations approximately equal to the pre-bloom levels. Over the period of the two blooms both Se(IV) and PSe show significant correlations with both POC and PON with $P < 0.01$ in all cases. After 7 April the nutrients lose their inverse correlation with the particulate phase.

The net effect of the two successive blooms is to cycle Se(IV) rapidly through the biota while apparently removing a small fraction of Se(VI). A single point sampling scheme prevents calculation of a comprehensive budget for selenium or elucidation of the ultimate fate of assimilated Se(VI). At the end of the overall sampling period Se(IV) levels are considerably enhanced over those in early January while Se(VI) values are virtually unchanged. Further interpretation of these observations in terms of cycling between the oxidation states is restricted by three considerations. First, towards the end of the sampling period (7 April) the nutrients lose their inverse correlation with the particulate phase—probably as a result of external inputs, for example, river run-off, which will similarly affect the selenium species. Second, the single point sampling scheme will certainly be unable to account for any vertical flux of selenium which may become important as stratification of the water column develops (historically in early April). Third, the fraction that we call Se(VI) will also by its methodological

definition¹⁰ include any labile dissolved organically bound selenium and thus represents an upper limit for Se(VI). Our own (unpublished) work, however, indicates that selenium in the dissolved organic fraction of seawater collected from Bedford Basin ranges between 87 and 102 pmol l⁻¹ and therefore represents only a minor part of the total dissolved element. In view of these limitations we can make no unique interpretation of the elevated post-bloom Se(IV) levels.

That Se(IV) during the two main blooms is removed from the dissolved phase to a much greater extent than Se(VI) is clearly shown by the inverse relationship of the Se(IV)/Se(VI) ratio (Fig. 2c) to biomass. Preferential assimilation of the lower oxidation state during algal blooms, the main components of which are the diatoms *Thalassiosira nodenskioldi* and *Chaetoceros septentrionalis* and the dinoflagellate *Gymnodinium* sp., could simply reflect a physicochemical selection process, based for example on ionic radius, resulting in non-biochemical incorporation of selenium into diatom tests. However, it is evident that selenium is an essential micronutrient for a wide variety of organisms¹², including phytoplankton¹³, and that the biochemically operational form is selenide. It is therefore probable that microorganisms will select the lowest available oxidation state because less chemical energy is then required for the reductive process linking selenium to organic molecules.

Maximal selenium levels in the particulate fraction precede PON, POC and chlorophyll peaks by 1–2 weeks. Although out of phase with biomass, the PSe maximum occurs when C:N ratios are lowest. This observation suggests a link between selenium metabolism and algal protein synthesis and is consistent with the observation that marine phytoplankton in culture incorporate the majority of assimilated selenium into protein¹⁴.

Temporal changes in the PSe:POC ratio (Fig. 1c) reveal the presence of two distinct pools of organic carbon with different selenium contents. The lower ratios of 3.9 and 5.4 pg per µg C associated with algal blooms reflect that of natural phytoplankton populations while the much higher ratio represents a background or non-living detrital pool. If algal carbon is taken to be 4.0% of wet weight¹⁵ then, using the derived PSe/POC ratios, the selenium content of phytoplankton is estimated to be between 160 and 220 ng per g wet weight. Selenium levels in organisms at higher trophic levels, usually reported to be in the low µg per g range, are significantly greater than the derived value for phytoplankton^{16–19}. For example, a concentration of 530 ng per g wet weight for Arctic pteropods (*Clione limacina*) has recently been obtained by the GLC technique used in the present study¹⁹. These observations suggest that selenium may be accumulated along the food chain.

If annual carbon fixation in the oceans is taken as 2.7 × 10¹⁶ g C yr⁻¹ and assuming that 93% of this material is recycled in the euphotic zone²⁰, the biologically mediated flux of selenium from surface water to deep water is estimated to range from 7.4 × 10⁹ to 1.0 × 10¹⁰ g Se yr⁻¹. In general terms our results provide direct evidence that biological activity in the euphotic zone can modify both the redox balance and the concentrations of a multiple oxidation state trace element. Similar biological effects may be of critical significance in the cycles of other trace elements in the oceans. Eutrophic coastal ecosystems should prove to be useful models in which new methods of trace analysis can be applied to examine such fundamental biogeochemical processes. While a more extensive sampling scheme will enable budgets to be calculated, a simple single point scheme as outlined here can provide a significant amount of information and at the same time lay a background against which more extensive studies can be designed.

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Microbial activity and bioturbation-induced oscillations in pore water chemistry of estuarine sediments in spring

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Temperature increases occurring during the spring in temperate shallow-water marine sediments lead to increased microbial activity, oxygen removal and a decrease in redox potential^{1,2}. These changes cause shifts in the relative importance of specific terminal electron acceptors used in bacterial respiration¹. The decrease in redox potential affects the physicochemical state of redox-sensitive elements such as iron and sulphur². In addition to changing sedimentary chemistry, these variations may affect the overlying water through changes in diffusional fluxes and other processes. Such biogeochemical processes are further complicated when the sediments are subjected to particle reworking and irrigation by bioturbating infauna^{3,4}. In an attempt to clarify the chain of sedimentary events occurring between the relatively oxidizing, inactive winter and the highly reducing, active summer, we examined several chemical and biological parameters in samples of estuarine sediment collected from February to July of 1980. Some of those data are presented here, and we report that the combined effects of increasing microbial activity and the onset of rapid bioturbation produced oscillations in the concentrations of pore water constituents in these sediments.

Sediment box cores were collected from a shallow-water site off the dock of the Jackson Estuarine Laboratory (JEL) in the Great Bay Estuary, New Hampshire. This location has been described previously⁵. Sediments at JEL consisted primarily of clay and silt-sized particles interspersed with fine sand. The upper 8–10 cm of these sediments are bioturbated actively from June to November⁶.

Horizontal sections (2 cm) of sediment were analysed for dissolved iron⁷ and dissolved organic carbon (DOC)⁸ (coefficients of variation of <1.0 and <0.5%, respectively) in conjunction with triplicate radiometric determinations of sulphate reduction^{5,9} and glucose turnover rates^{5,10}. All sediment manipulations and pore water extractions and filtrations (0.4 µm Nuclepore) were carried out under N₂ to prevent oxidation artefacts¹¹.

Temporal changes in the chemical and bacteriological parameters at JEL are presented in Fig. 1. Data points represent averages of the upper 6 cm of sediment to demonstrate the breadth of the variations noted. Dissolved iron concentrations varied more with depth than the other parameters. However, similar temporal trends were noted at each depth³.

During winter, sulphate reduction rates were slow, <50 nmol per ml per day. Glucose turnover rates were relatively slow and reported as the sum of radiocarbon recovered as carbon dioxide, particulate material (microbial biomass) and ether-soluble dissolved end products⁵. Iron was nearly absent from solution as a result of its oxidation and precipitation¹². The upper 3–4 cm of these sediments were orange-brown in colour. A previously reported winter increase in sulphate/chloride ratios¹³ demonstrated the generation of sulphate in JEL sediments from the oxidation of iron monosulphides. Concentrations of acid-volatile sulphides in the upper 2 cm increased 12-fold between April ($4.0 \mu\text{mol ml}^{-1}$) and August ($48.0 \mu\text{mol ml}^{-1}$) 1980⁵. Despite the relatively oxidizing state of these surface sediments during winter, sulphate reduction was detected throughout, although at low levels. Winter DOC concentrations were high, an unexplained but common phenomenon in these pore waters (M.E.H., W.H.O. and W.B.L., in preparation).

As the sediment temperature increased in March and April, there was an increase in heterotrophic activity. This was shown, indirectly, by the dissolution of iron. The relative biotic and abiotic contributions to this iron reduction are unknown¹⁴. Glucose turnover rates increased; however, without knowledge of the glucose pool size, this increase is not positive evidence of increased heterotrophy. DOC was consumed rapidly during this period. We have yet to determine if this DOC decrease is due to uptake by bacteria, abiotic mechanisms or a combination of both. In any event, this early spring interval is characterized by rapid changes in sedimentary chemistry.

Sulphate reduction rates remained slow during April even though the temperature had increased. Howarth and Teal¹⁵ reported a similar finding for salt marsh soils.

In May, sulphate reduction activity increased. Dissolved iron was partially removed from solution, undoubtedly due to ferrous sulphide precipitation. DOC concentration increased as a result of sulphate reduction processes¹⁶.

Bioturbation activity increased rapidly in late May–early June (arrows, Fig. 1). This increase was reflected by dramatic changes in the visual and textural character of the sediment such as increased porosity and homogeneity. In addition, previous seasonal X-ray analyses demonstrated this increase in macrofaunal activity⁶. The start of active bioturbation was accompanied by a tripling of sulphate reduction rate, a doubling of the rate of glucose turnover, a five-fold increase in the concentration of dissolved iron and a decrease in DOC.

The dominant active macroorganism in JEL sediments in June is the capitellid polychaete *Heteromastus filiformis*¹⁷. As discussed by others^{3,4,18,19}, bioturbation can increase microbial activity through reworking and irrigation activities while decreasing pore water constituents through facilitated advection. Anaerobic metabolism was enhanced at JEL by the macrofauna-mediated transport of organic material into deeper anoxic regions. The magnitude of this enhancement and other bioturbation-mediated aspects of these sediments will be reported elsewhere.

During summer, bioturbation remained active. DOC concentrations continued to decrease even though anaerobic microbial activity was rapid. Dissolved iron concentrations decreased into July yet remained relatively high at 7–8 mg l^{-1} .

Although bioturbation seemed to enhance sulphate reduction and therefore sulphide production, iron remained in solution during the summer. For comparison, we measured these same parameters in sediments from another site in the Great Bay Estuary located near the mouth of the Squamscott and Lamprey Rivers (SQUAM). These sediments consisted of similar size particles as JEL. However, seasonal X-ray analyses demon-

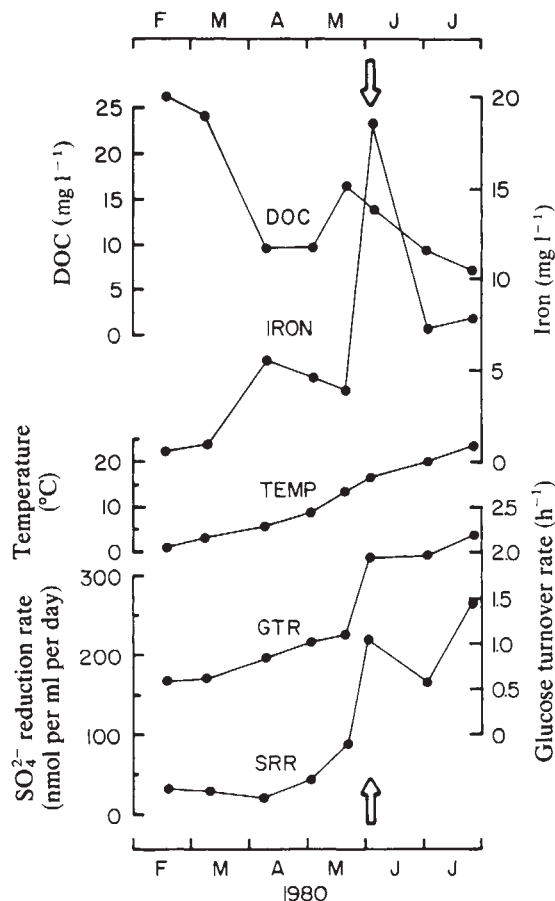


Fig. 1 Variations in sulphate reduction rate (SSR), glucose turnover rate (GTR), temperature (TEMP), dissolved iron and dissolved organic carbon (DOC) in the upper 6 cm of sediments at JEL. Values are averages of replicate determinations from three 2-cm horizontal sections of sediments. Arrows indicate when the start of active bioturbation was observed.

strated that SQUAM sediments are not significantly bioturbated⁶. Dissolved iron concentrations in SQUAM sediments increased from 0.2 to 2.5 mg l^{-1} in May, while DOC decreased from 18 to 10 mg l^{-1} . However, with the onset of sulphate reduction and lack of bioturbation, iron concentrations decreased to near zero in July while DOC increased to 20 mg l^{-1} . These summer values occurred even though sulphate reduction was less than one-third as rapid as in JEL sediments⁵. Therefore, the net effect of bioturbation was the maintenance of high dissolved iron concentrations in JEL sediments even though the removal of DOC was enhanced. As discussed by Aller³, bioturbation may affect iron speciation by the transport of surface Fe^{3+} and the oxidation/reduction of iron sulphides during reworking and irrigation. However, unlike Aller's³ sediments, which were bioturbated most actively in later summer, bioturbation in the JEL sediments began rapidly in June and overwhelmed the changes in iron and DOC concentrations previously initiated by increased sulphate reduction.

Lyons *et al.*² provided evidence that, during April, a large portion of the dissolved iron in JEL sediments is organically associated. This association may have aided in preventing the removal of iron as a sulphide precipitate. However, the pore water iron concentration in June was extremely high (Fig. 1) and the molar ratio of DOC to iron was only 4.1. This ratio was 2.2 in the top 2 cm of the sediment. Hence, organically associated iron is not a likely mechanism for maintaining this quantity of iron in solution in the presence of rapid sulphide production.

The net rate of iron dissolution between late May and early June at JEL was 11 nmol per ml per day (Fig. 1). As these sediments were fairly homogeneous and highly reducing, the

gross rate of iron reduction probably exceeded the sulphide production rate of 100–200 nmol per ml per day. Because other metals often are associated with iron oxyhydroxides²⁰ or may behave similarly to iron, this spring transition period may profoundly affect the speciation and mobility of other elements.

The combined effects of increased microbial activity and bioturbation-mediated sediment reworking, irrigation and enhanced anaerobic processes caused an oscillation in the pore water concentrations of iron and DOC. In addition, dissolved phosphate, ammonium and alkalinity concentrations varied similarly to DOC⁸. The amplitude and wavelengths of these temporal changes would be expected to vary yearly depending on differences in temperature regimes and weather events. Weather-related variations have been noted⁵ and seem to be the result of the removal of infauna by storms. Oscillations in the concentrations of pore water constituents may affect the diffusional flux of material across the sediment–water interface and when combined with bioturbation-enhanced fluxes may have a profound effect on sediment and overlying water processes. Most importantly, metals may undergo rapid speciation changes which could influence the flux of pollutants into the water column and the distribution of sedimentary minerals.

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Reappraisal of sea-ice distribution in Atlantic and Pacific sectors of the Southern Ocean at 18,000 yr BP

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The question of the distribution of winter and summer sea-ice cover during the last glacial maximum (18,000 yr BP) is important in climatic modelling of the glacial world. We have now compared satellite-derived 5-yr monthly averages of per cent summer sea-ice cover in the Atlantic and Pacific sectors of the Southern Ocean with the distribution of reported sedimentological indicators of sea-ice cover at the sediment–water interface. These data strongly suggest that sediment type cannot be used to identify summer sea-ice limits, neither in Recent sediments nor in sediments of the last glacial maximum. Rather, the data seem to indicate that sediment type can be used to identify spring sea-ice limits for these two time intervals.

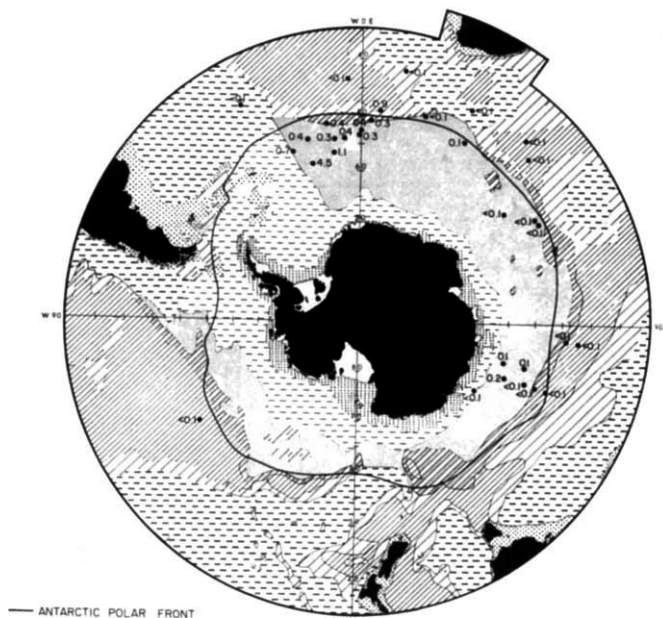


Fig. 1 Distribution of surface sediments around the Antarctic continent. Sediment types from the continent northward are as follows: parallel rows of dots = shelf and coastal deposits; light parallel dashed lines = siliceous silty clay and clayey silt; fine dots = siliceous ooze; oblique solid lines = calcareous ooze; oblique solid lines alternating with fine dots = siliceous and calcareous ooze. Numbers refer to percentage coarse fraction. Data are from ref. 9.

Attempts to define past sea-ice boundaries in the Antarctic began with Philippi¹ but considerable refinements have since been added^{2–5}. Lozano and Hays⁶ and Hays *et al.*⁷ were the first to place the various sediment types within a good chronostratigraphical context. They were able to show that the thin veneer of diatomaceous ooze found in the Southern Ocean was Holocene in age while the underlying silty diatomaceous clay represented glacial conditions. These alternating sediment types were directly linked to the oxygen isotope record and permitted Hays *et al.*⁷ and Cooke and Hays⁸ to devise a chronostratigraphic framework for late Quaternary sediments of the Southern Ocean. These workers have argued that the boundary in surface sediment between the silty diatomaceous clay and the diatomaceous ooze represents the northern edge of summer sea ice (Fig. 1). Further, they argue that this boundary at 18,000 yr BP represents the northern limit of summer sea ice during a glacial maximum. As might be expected, this boundary is considerably north of the present-day boundary and closely approximates present-day winter sea-ice limits. The presence of diatoms within the silty diatomaceous clay is attributed both to leads which opened in the summer sea-ice, resulting in higher phytoplankton productivity, and to occasional summers when sea ice melted back almost to the continent (ref. 8 and J. D. Hays *et al.*, in preparation).

Here we suggest an alternative interpretation of the sediment analysis of Cooke⁹ and Cooke and Hays⁸. We use modern-day sea-ice distribution and compare it with sediment distribution (and particularly sediment type boundaries) in surface sediments and at 18,000 yr BP⁹. Sea-ice cover, derived predominantly from satellite imagery¹⁰, was averaged monthly over a 5-yr period (1973–77) for both the Atlantic and the Pacific sectors (Fig. 2). These data show a seasonally fluctuating ice front with a low percentage of sea-ice cover near the ice margin and higher values ranging between 75 and 100% some 6–7° behind the ice front. The lower values encountered in higher latitudes near the continent represent polynyas where high winds have pushed the sea ice away from the shore and maintained open water, sometimes well into the winter and in early spring.

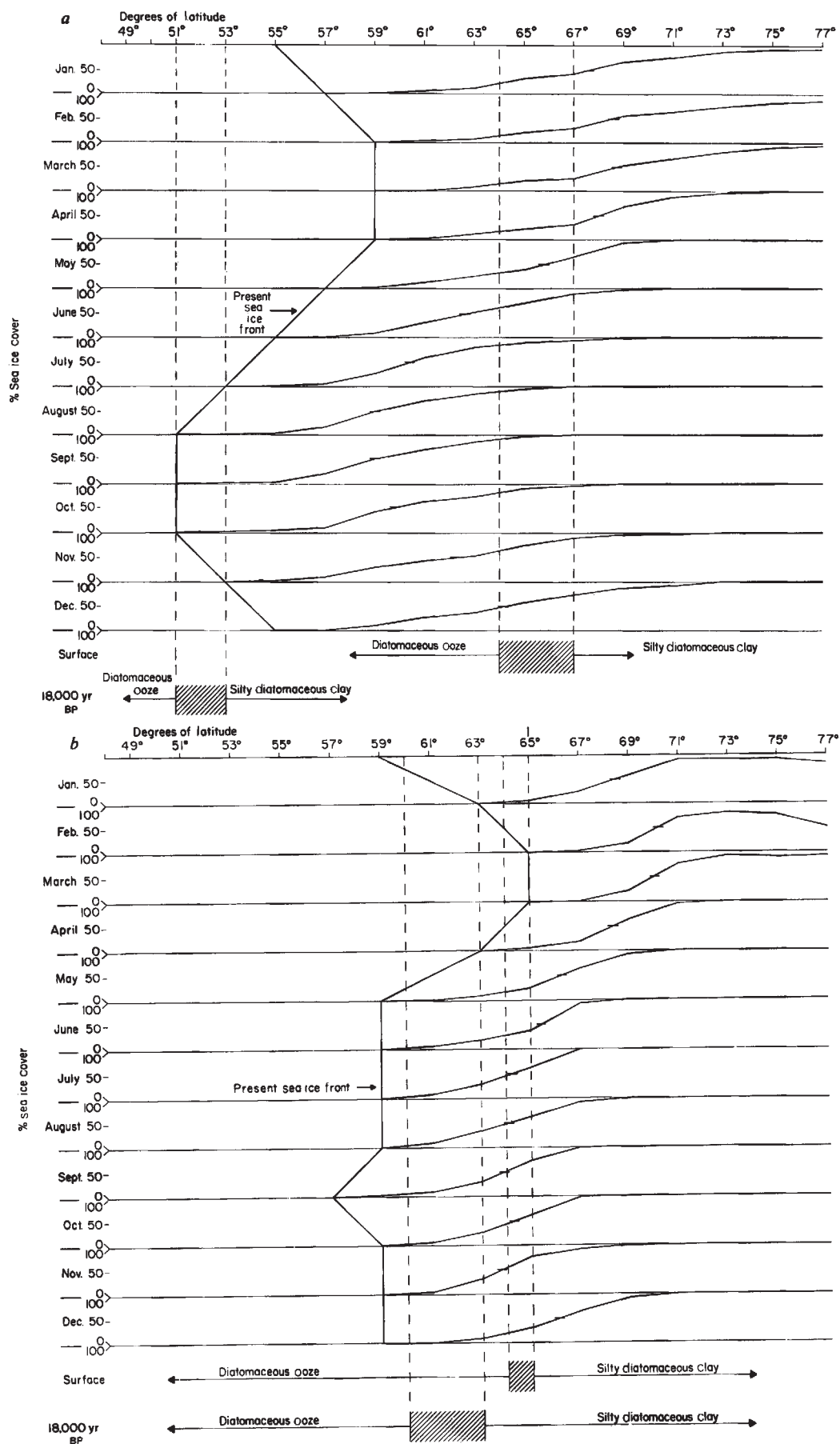


Fig. 2 Satellite-derived 5-yr monthly averages of per cent sea-ice cover for each 2° of latitude in the Pacific (a) and Atlantic (b) sectors of the Southern Ocean. Data on sediment distribution in Recent and at 18,000 yr BP are taken from ref. 9.

Figure 2 also shows the present-day northern boundary between silty diatomaceous clay and diatom ooze⁹. This boundary is rather irregular but is between 64° and 65° in the Pacific sector and 64° and 67° in the Atlantic sector. If this boundary is projected up through the diagram, it is seen that in the Pacific the range of sea-ice cover is 0–0.1% during the months of February to March. In the Atlantic, on the other hand, the sea-ice concentration during those 2 months ranges over 15–30%. A sea-ice concentration of 0.1% seems insufficient to produce a silty diatomaceous clay on the sea floor. Equally doubtful is the notion that 15–30% sea-ice cover is enough to produce such a sediment type. It seems, therefore, that this sediment boundary cannot represent summer sea-ice limits.

Although we believe that this approach is better than those previously attempted, there are some obvious weaknesses in it. Kukla and Gavin¹⁰, for example, have recently shown that in the 1930s, summer ice conditions were heavier than at present. However, this translates into a change in the position of the sea-ice edge of the order of 1–2° of latitude. Further, as Kukla and Gavin¹⁰ point out, there are periodic variations ranging from a few years to tens of years in duration in oceanic circulation and these would probably affect sea-ice distributions. Heap¹¹ also reported oscillations in ice extent in the Weddell Sea and Palmer Peninsula. Finally, Kukla¹² and Kukla and Gavin¹⁰ report extensive spring sea ice in 1972 and 1973 and again in 1981, with declining spring sea-ice cover in the intervening years. These data suggest, therefore, that the use of sea-ice cover averaged over a 5-yr period or better may be adequate for comparison with surface sediment data.

If not summer sea-ice limits, what event does this sediment boundary record? Figure 2 shows that the biggest drop in ice cover overlying this sediment boundary occurs in the late spring (November–December). In the Pacific, the change is from 60–80% in November to ≤5% in January. In the Atlantic, the change is less dramatic, but the ice cover drops from as much as 90% in November to slightly more than 40% in January. The fact that this ice lingers on into late spring before retreating produces several results. Most important is the fact that the presence of the ice inhibits open-ocean diatom productivity. There are, of course, diatoms living and growing in the underside of the sea ice, but this production is considerably less than in the open ocean. The diatom growing season begins in November north of the Polar Front and moves southward as the spring and summer seasons progress. As has been shown^{13,14}, the southward progression of this productivity maximum is heavily dependent on the melt back of the ice. Thus, sea ice can act to inhibit diatom productivity.

A second important point is the origin and distribution of the silt and clay size particles associated with diatoms in the sediment. Spring peaks in aerosol concentration and condensation nuclei have been recorded in the Antarctic atmosphere^{15,16}, suggesting a Southern Hemisphere similarity with that of Murozumi *et al.*¹⁷, who found terrestrial dust concentrations of Greenland spring snows to be three times larger than those of other seasons. It seems that whereas this dust falls on the open ocean and is dispersed, the sea ice acts as a collector and selectively dumps it in the late spring and summer. This point, combined with the fact that the sea ice may act as an inhibitor of diatom productivity until well into the growing season, seems to be a reasonable mechanism for producing a silty diatomaceous clay on the sea floor.

The lack of additional data both of ice cover and of surface and 18,000 yr BP sediment distribution prevent us from further speculation. However, we feel that the true summer ice limit during the last glacial maximum is more appropriately placed south of all data points. This means that this limit has not been defined for the last glaciation and is a suitable target for study by palaeoceanographers.

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De-A-steroid ketones and de-A-aromatic steroid hydrocarbons in shale indicate a novel diagenetic pathway

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Recent investigations of Cretaceous black shales have led to the identification of several hitherto unknown steroidal compounds^{1,2}, thus providing information on some diagenetic pathways of steroids. We report here the identification of ring A-degraded steroids in a Cretaceous black shale from the Livello Bonarelli, a carbonate-poor interval deposited at the Cenomanian–Turonian boundary. The ketone fraction of the extract contains a series of compounds which we have identified as 10 α (Me)-de-A-cholestan-5-one and homologues. The presence of mono- and diaromatic de-A-steroid hydrocarbons in the aromatic fraction of the extract is suggested. The occurrence of these degraded steroids can be explained by a degradation pathway similar to that suggested for 3-oxygenated triterpenoids such as β -amyryn and lupeol^{3–7}.

The shale sample was taken from an exposure (43°33' N, 12°41' E) near the cemetery of Moria, a small village near Cagliari, province of Pesaro and Urbino, Italy⁸. The sediment, which has an organic carbon content of 13.3%, was Soxhlet-extracted with toluene/methanol (1:3 v/v; 20 h). The hexane-soluble part of the extract was separated by column chromatography over silica gel. Elution with 50-ml portions of hexane containing increasing amounts of diethyl ether (0, 1, 3, 5, 7, 10, 20%)

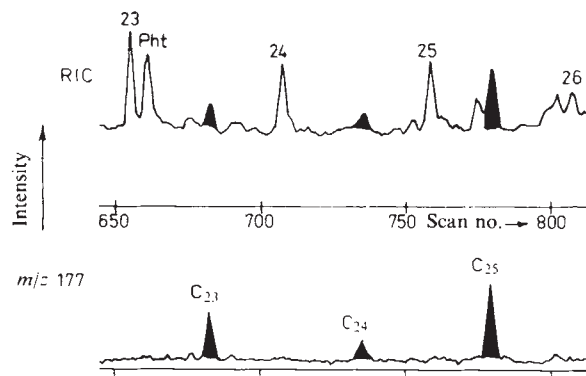


Fig. 1 Part of the reconstructed ion chromatogram (RIC) and part of the mass chromatogram of m/z 177. Numbered compounds in the RIC are n -alkan-2-ones. Pht, phthalate. Temperature programme: 150–300 °C (4 °C min⁻¹).

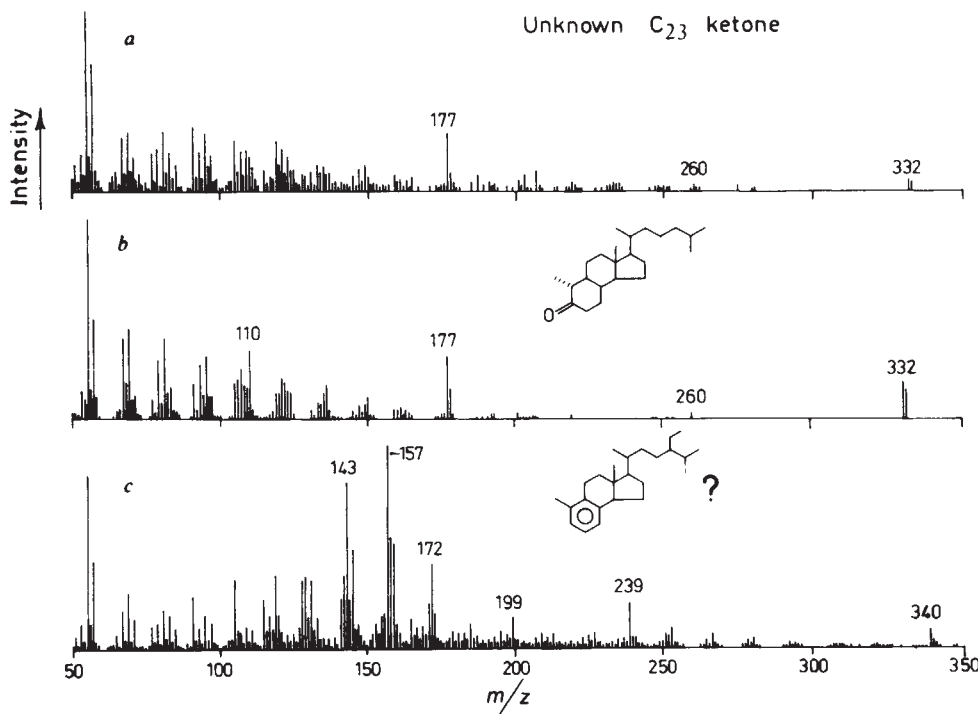


Fig. 2 Mass spectra of *a*, unknown C_{23} ketone; *b*, synthetic $10\alpha(\text{Me})\text{-de-A-cholestan-5-one}$; *c*, C_{25} monoaromatic de-A-steroid hydrocarbon (tentative identification). Spectra *a* and *c* comprise contributions from other compounds.

yielded several fractions including aromatic hydrocarbons (1 and 3% diethyl ether) and ketones (7 and 10% diethyl ether). Gas chromatography-mass spectrometry was performed on a Varian 3700 gas chromatograph equipped with a 25-m fused silica column coated with CP-sil-5, coupled to a MAT 44 quadrupole mass spectrometer operating at 80 eV (mass range m/z 50–500, cycle time 2 s).

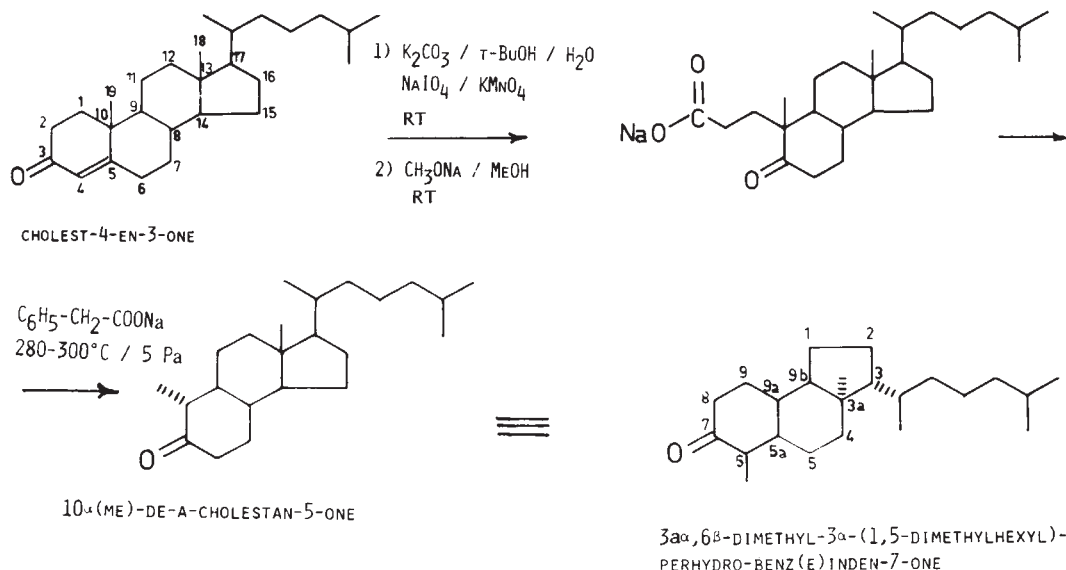
The ketone fraction contains several series of n - and isoprenoid alkan-2-ones, steroidal and hopanoid ketones, and a series of three compounds whose mass spectra exhibit a characteristic peak at m/z 177 (Fig. 1). The distribution pattern of these compounds is similar to that often encountered with steroidal compounds. As the main fragment ion at m/z 177 and the molecular ions at m/z 332 (Fig. 2*a*), 346 and 360 of the unknown compounds were 54 mass units lower than the corresponding ions in the mass spectra of C_{27} , C_{28} and C_{29} cholestan-3-ones (m/z 231, 386, 400 and 414 respectively), we assumed that we were dealing with a series of de-A-steroid ketones. To test this hypothesis we synthesized de-A-cholestan-5-one ($3\alpha,6\beta$ -dimethyl- 3α -(1,5-dimethylhexyl)-perhydrobenz(e)inden-7-one) starting from cholest-4-en-3-one (Fig.

3)^{9–11}. During the removal of ring A the orientation of the C-19 methyl group changes from β to the more stable α configuration¹⁰.

The synthetic compound co-eluted with the unknown C_{23} compound on two stationary phases (OV 101 and SE 52). On the basis of this observation and a comparison of the mass spectra (Fig. 2*a,b*), we concluded that the unknown C_{23} compound was identical to $10\alpha(\text{Me})\text{-de-A-cholestan-5-one}$. The unknown C_{24} and C_{25} compounds are probably the 24-methyl- and 24-ethyl-substituted homologues.

The aromatic fraction of the extracts contains C_{23} , C_{24} and C_{25} compounds whose mass spectra resemble those of ring A monoaromatic steroid hydrocarbons [4-methyl-24-ethyl-19-nor-cholesta-1,3,5(10)-triene: m/z 394 (M^+ , 85%), 253 (M^+ -side chain, 5%), 226(13%), 211(100%), 197(8%), 158(66%), 157 (32%)]¹, but with a shift of 54 mass units for the prominent ions (Fig. 2*c*). Therefore, we may be dealing with a series of de-A-ster-5,7,9(10)-trienes ($3\alpha,6\beta$ -dimethyl- 3α -(1,5-dimethylhexyl)-2,3,3a,4,5,9b-hexahydrobenz(e)indenes). Also present in the aromatic fraction are compounds with mass spectra having a base peak at m/z 195 and weak molecular

Fig. 3 Preparation of $10\alpha(\text{Me})\text{-de-A-cholestan-5-one}$ from cholest-4-en-3-one^{9–11}. RT, room temperature.



ions (among others at m/z 322 and 336). These compounds could be diaromatic de-A-steroid hydrocarbons. Unambiguous identification of the mono- and diaromatic compounds will only be possible after synthesis of the proper reference compounds.

As ring A-degraded steroidal compounds have not been reported in living organisms, it seems likely that they are formed by diagenetic degradation of steroids. This degradation could proceed along the pathway proposed for 3-oxygenated triterpenoids such as β -amyrin and lupeol³⁻⁷. Cleavage of the C-3/C-4 bond may occur through a photochemical or microbial oxidation yielding 3:4 seco acids⁸. Further steps may involve the

removal of the ring A remains, yielding de-A-triterpanes⁷. The formation of di-^{4,5} and triaromatic³ de-A-triterpenoid hydrocarbons is also possible. The occurrence of ring A-degraded steroids in the sediment investigated is compatible with the degradation pathway described. The likelihood of this pathway in the case of steroids would be greatly enhanced by the identification of 3:4 seco steroidal acids in recent sediments.

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Pseudocholinesterase staining in the primary visual pathway of the macaque monkey

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Cholinesterases in brain tissue are divided into two main classes: 'true' cholinesterases (acetylcholinesterase, AChE, EC 3.1.1.7), which preferentially hydrolyse the neurotransmitter acetylcholine; and 'pseudocholinesterases' (for example, butyrylcholinesterase, BuChE, EC 3.1.1.8), which preferentially hydrolyse higher choline esters¹. AChE is found at peripheral and central cholinergic synapses, is known to be the degradative enzyme of the cholinergic mechanism and may also have other functions in the central nervous system¹⁻³. In contrast to AChE, the pseudocholinesterases have been assigned no certain function in neural transmission and initially were thought to occur mainly in neuroglia, Schwann cells and vascular endothelia^{1,4}. Pseudocholinesterase activity has since been found in neurones and neuropil in several brain regions^{1,4,5}, however, and in the superior cervical ganglion, BuChE has been localized to postsynaptic membranes⁶ and shown to exist in stable molecular forms corresponding to each of the known molecular forms of true cholinesterase⁷. These observations have led to the alternative interpretations that the pseudocholinesterases are either metabolic precursors of AChE⁸ or that they have functions closely related to those of AChE^{5,7} while being independent of true cholinesterases. We have directly compared the distributions of BuChE and AChE in the central visual pathway of the primate, a neural system in which anatomical and functional compartmentalization is well known⁹. We demonstrate here that the histochemical localization of pseudocholinesterase rivals that of AChE in terms of specificity, and that BuChE is independent of AChE both in its normal distribution in the lateral geniculate body and striate cortex and in the response it shows to eye enucleation. We conclude that BuChE or its endogenous substrate may be a neuroactive substance in the primate brain.

In the brains of nine adult rhesus macaque monkeys, we studied the distributions of BuChE and AChE in serially adjoining sections through the lateral geniculate body and pulvinar nuclei of the thalamus and through the striate and peristriate cortex. We related these, in turn, to the distribution of cytochrome oxidase, a histochemical marker for modular tissue arrangements in the visual cortex¹⁰⁻¹². Three of the monkeys had undergone unilateral (left) eye enucleation, 10, 15 and 29

days before death. All brains were fixed by vascular perfusion with 4% paraformaldehyde in 0.1 M PO₄ buffer and cut at 30-50 μ m on a freezing microtome. For the cholinesterase histochemistry we followed modified thiocholine protocols¹³: BuChE sections were incubated with butyrylthiocholine iodide as substrate in the presence of 10⁻³-10⁻² M BW284c51 (Burroughs Wellcome), an AChE inhibitor; AChE sections were incubated with acetylthiocholine iodide as substrate in the presence of a saturated solution of ethopropazine (Parsidol, Warner Chilcott), a pseudocholinesterase inhibitor; and control sections were incubated with either substrate in the presence of 1 mM eserine, a general inhibitor of cholinesterases. Incubations were carried out at room temperature and at 4°C for times ranging from 13 h to 13 days for the BuChE and 4 h to 10 days for the AChE. Cytochrome oxidase sections were prepared according to the protocol described by Wong-Riley¹².

In the normal monkeys there was a striking compartmentalization of BuChE activity in relation to known functional subdivisions of the visual thalamus and cortex. First, the enzyme stain sharply distinguished between the parvocellular and magnocellular layers of the lateral geniculate body: the parvocellular layers were rich in BuChE, the magnocellular layers were nearly devoid of staining (Fig. 1a). Second, the several subnuclei of the pulvinar were stained in a non-uniform way, as we will describe separately. Finally, there was a detailed patterning of BuChE staining in the striate cortex which set it off from surrounding areas (Fig. 2a): layer 4C α was most densely stained and thin dark laminae appeared at the borders of layer 5. Layer 4C β , at least at its base, was only very weakly stained. In the supragranular layers BuChE activity was concentrated in 100-300 μ m wide patches which in tangential sections formed a partly connected gridwork. As shown in Fig. 2, the spacing of the BuChE patches was similar to that of the patches of cytochrome oxidase visible in serially adjoining sections (300-500 μ m centre-to-centre) but the relationship between the two sets of patches was not clear because they were, in the case material, instances of alignment, complementarity and apparent lack of correlation of the two patterns. In the infragranular layers there was also clear periodicity in the BuChE and, especially in layer 6, some prominent BuChE-positive neurones. Vascular staining was notable in the BuChE sections because the walls of the microvessels, but not the larger vessels, showed intense enzyme activity.

Neither in the thalamus nor in the cortex did the distribution of AChE match that just described for BuChE. In the lateral geniculate body all of the cell layers were stained for AChE, the magnocellular layers more intensely than the parvocellular¹⁴ (Fig. 1b). This pattern closely matched that of cytochrome oxidase. Zones of high and low AChE activity also appeared in the pulvinar, but were not sharply different from the BuChE-positive subdivisions. In the striate cortex AChE staining was most pronounced in layers 4A and both 4C α and 4C β , and was

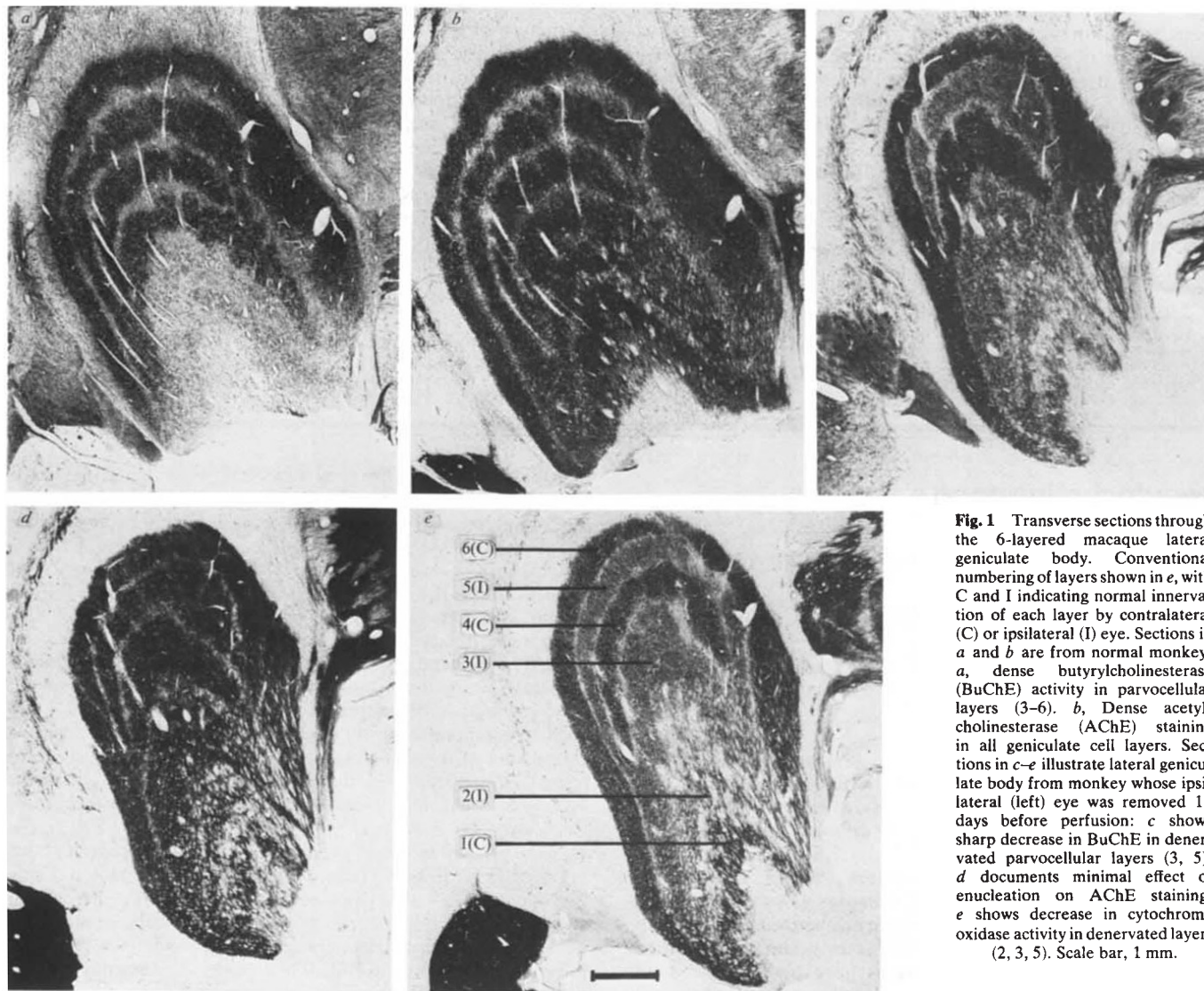


Fig. 1 Transverse sections through the 6-layered macaque lateral geniculate body. Conventional numbering of layers shown in *e*, with C and I indicating normal innervation of each layer by contralateral (C) or ipsilateral (I) eye. Sections in *a* and *b* are from normal monkey: *a*, dense butyrylcholinesterase (BuChE) activity in parvocellular layers (3-6). *b*, Dense acetylcholinesterase (AChE) staining in all geniculate cell layers. Sections in *c-e* illustrate lateral geniculate body from monkey whose ipsilateral (left) eye was removed 15 days before perfusion: *c* shows sharp decrease in BuChE in denervated parvocellular layers (3, 5); *d* documents minimal effect of enucleation on AChE staining; *e* shows decrease in cytochrome oxidase activity in denervated layers (2, 3, 5). Scale bar, 1 mm.

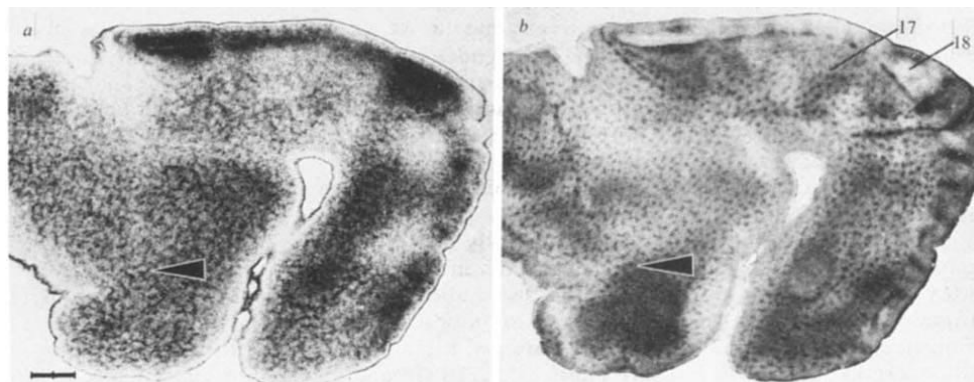
strong in layers 1 and 6 (see ref. 15). Patches were present in the supragranular layers in most preparations and, in sections studied in detail, lay in register with the BuChE patches. They were fuzzy and difficult to see, however, even after prolonged incubations.

Unilateral eye removal resulted in marked changes in these cholinesterase patterns but did not influence the two enzymes in the same way. In the lateral geniculate body there was a blanching of BuChE stain in the parvocellular layers innervated by the enucleated eye (Fig. 1*c*) but the AChE patterns in the geniculate appeared nearly normal, at most a slight weakening

being visible in the denervated layers (Fig. 1*d*). The cytochrome oxidase was decreased in the denervated layers (Fig. 1*e*).

Although AChE activity did not change greatly in the geniculate, it did in the striate cortex, where, as shown for the 10-day eye enucleate in Fig. 3*a*, stripes of high AChE activity alternated regularly with zones of low AChE activity in layer 4C. Changes in BuChE staining were more difficult to detect in transverse sections, but in tangential sections through the base of layer 4 (Fig. 3*b*) and in favourable transverse sections, periodically arranged thin dark stripes could be seen. Even 10 days after enucleation, there also were subtle periodic variations in the

Fig. 2 Adjacent tangential sections cutting through supragranular layers of the visual cortex in a normal macaque monkey. *a*, Supragranular gridwork of BuChE activity in area 17. *b*, Patches of cytochrome oxidase activity in supragranular layers. Tips of arrows touch blood vessel running through the two sections. In this pair of sections many BuChE patches avoid cytochrome oxidase patches. The border between areas 17 and 18 is shown to right and top. Note that both BuChE and cytochrome oxidase are distributed unevenly in area 18 as well as in area 17. Sections 50 μ m thick; scale bar, 2 mm.



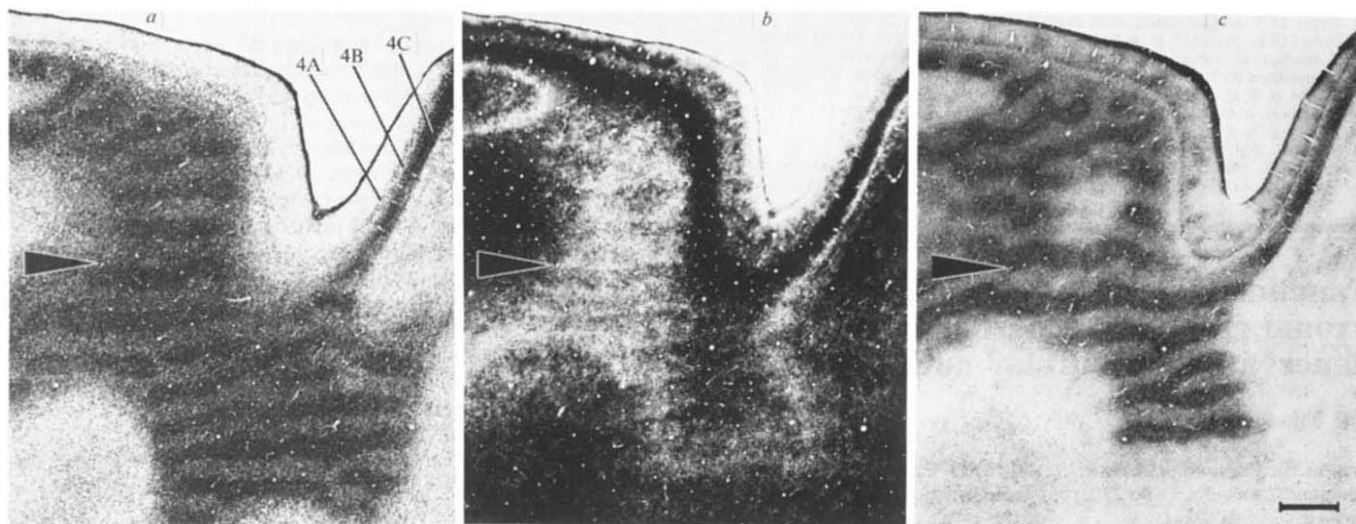


Fig. 3 Serially adjoining 50- μ m thick tangential sections through area 17 of a macaque monkey whose ipsilateral (left) eye was enucleated 10 days before perfusion. *a*, AChE activity; *b*, BuChE activity; *c*, cytochrome oxidase activity. Sections illustrate that, as a result of the eye enucleation, stripes form in layer 4 in all three stains. Similar stripes appeared on the contralateral side. Arrows indicate position common to all sections, located by using blood vessels as fiducial markers. Alignment of the sections shows that the AChE-rich and BuChE-rich stripes in *a* and *b* are in register but that they correspond to the stripes poor in cytochrome oxidase activity in *c*. At the upper right, the plane of section is oblique to the cortical layers, which are numbered according to the scheme of Lund¹⁸. Scale bar, 1 mm.

intensity of Nissl staining in layer 4C and in the supragranular layers (not illustrated; see ref. 16). Alignment of serial sections such as those shown in Fig. 3 demonstrated that the dark BuChE stripes were in register with the dark AChE stripes (and also with the zones of darker Nissl stain). Remarkably, the stripes of high AChE and BuChE activity coincided with stripes of low cytochrome oxidase activity, known from previous work to represent the enucleated eye¹⁰. This finding (see Fig. 3*a-c*) will be described further elsewhere.

The present study demonstrates that BuChE is a sensitive marker for functional subdivisions in the primate visual system. In the lateral geniculate body, high BuChE activity singles out the layers reported to have physiological properties of the X (colour-opponent) type¹⁷. In the cortex, BuChE activity patterns distinguish area 17 from area 18 and within area 17 delimit distinct sublaminae as well as periodic elements in the supragranular and infragranular layers. It is unlikely that the geniculate and cortical patterns of BuChE activity denote a single functional system. For example, the BuChE-rich parvocellular layers of the geniculate project mainly to layers 4A and 4C β ¹⁸, yet in the striate cortex BuChE in layer 4 is densest in the magnocellular-recipient 4C α . Nor is the supragranular patchwork of BuChE activity in area 17 clearly related to geniculate innervation. It did not undergo changes with eye enucleation comparable to those observed in the cytochrome oxidase sections and was not even always aligned with the cytochrome oxidase grid: in some serial sections the two sets of patches were in complementary positions. Thus, in the striate cortex, BuChE may mark fibre systems intrinsic to area 17¹⁹ or systems originating in extrastriate cortex¹⁸ or in the pulvinar²⁰. By virtue of branching of the patches, the BuChE gridwork showed a striking resemblance to 2-deoxyglucose patterns illustrated by Hubel *et al.*²¹ and others²²⁻²⁴. Identification of the BuChE-containing elements may therefore help resolve uncertainty as to the origin of the heightened metabolic activity in the supragranular grid^{10,24,25}.

In the lateral geniculate body the BuChE pattern seemed clearly associated with the primary visual pathway and the systematic decreases in BuChE activity on denervation suggest that BuChE may be a highly specific marker for retinal axon terminals or the neurones which they innervate. An alternative explanation is that BuChE is a marker of functional activity as cytochrome oxidase is thought to be¹², but this is made unlikely by the fact that BuChE activity in the cortex did not decrease in the regions representing the removed eye.

Both the specificity in the distribution of BuChE and its independence of AChE levels emphasize the possibility that the pseudocholinesterases or their substrates may be neuroactive substances in the central nervous system. The present findings suggest that the primate visual pathway may serve as a valuable system in studies designed to identify such endogenous substrates, which, based on previous reports, could include neuropeptides or compounds related to γ -aminobutyric acid metabolism^{5,26}.

In addition to these findings on the distribution of BuChE, a corollary observation of importance in its own right was the sensitivity of cortical AChE to eye enucleation. AChE in the brain stem is known to derive from several sources^{5,15} but in most of the neocortex AChE levels have been reported to depend on the integrity of the nucleus basalis of Meynert, which as part of the substantia innominata is thought to provide the main cholinergic innervation of the neocortical parts of the cerebral hemispheres^{5,27,28}. The implication of the present study is that the cortical fibre systems delimited by the presence of AChE are probably multiple, including at least some that are dependent on activity in the main sensory pathways and possibly, more generally, on thalamic innervation.

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Functional reconnections without new axonal growth in a partially denervated visual relay nucleus

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Physiological mapping of the retino-geniculate projections in adult cats which had previously undergone partial retinal lesioning have demonstrated a delayed spread of excitation into regions initially deafferented¹. Anatomical studies in the adult cat^{2–5} and monkey⁶ have, however, not revealed any new axonal growth. The present results demonstrate that the lesion-induced functional reorganization is brought about not by new axonal growth but probably by local changes at the periphery of the dendrites.

In a combined electrophysiological and autoradiographic study of the partially deafferented lateral geniculate nucleus, extracellular recordings were obtained from the light-excitable cells closest to deafferented geniculate zones. The cells were marked by electrolytic lesions, and ³H-proline was then injected into the damaged eye to trace the distribution of surviving retinal afferents to the geniculate cells. After short survival times light-excitable and distribution of autoradiographic label corresponded, but after survival times longer than 30 days light-excitable cells were detected in the unlabelled regions for a distance of up to 150 μ m beyond the boundaries of the autoradiographically labelled zones.

Single-cell recordings obtained from the lateral geniculate nucleus (LGN) of adult cats with long survival times after photocoagulation in the nasal retina have revealed characteristic topographical changes in the border region between normal innervation and deafferentation¹. At 1–20 days after retinal lesions normal retino-geniculate projection columns⁷ were

found at the borders of deafferented regions in layer A (Fig. 1a). After survival times exceeding 25 days, excitation from parts of the retina adjacent to the lesions could be detected in the normal topographical location and also up to 200 μ m inside the initially deafferented zones of layer A¹. This was demonstrated by making recordings from cells in layer A with receptive fields at the border of the retinal lesions which were displaced by up to 4.5° of visual angle with respect to the normal receptive field positions of layer A₁ cells recorded during the same vertical penetrations. In layer A the respective projection columns had expanded asymmetrically into the deafferented region (Fig. 1b).

The time course with which the displaced receptive fields of layer A cells developed was similar to that observed in anatomical studies of axonal sprouting in subcortical structures of the cat central nervous system^{8–10}, but no indications of any long-range axonal sprouting had been found in the adult cat LGN after either monocular enucleations^{2,3} or focal lesions of the retina⁴, while axonal sprouts of 100–200 μ m had not been ruled out. Because sprouting in this range would explain our neurophysiological observations, we tested this possibility in the range of 200 μ m with an autoradiographic experiment.

In five cats a narrow normal retinal strip of constant width (6–8°), bordered by photocoagulator lesions, was created in two stages so that the upper part was bordered by long-established (90–160 days) and the lower part by recent (6 days) lesions (Fig. 1c). The high-intensity lesions destroyed all excitable layers of the retina. In the final experiment, electrolytic lesions were made in the LGN to mark the regions of long- and short-term deafferentation. ³H-proline was injected into the eye to label the axons projecting to the LGN. This allowed an autoradiographic comparison of the projections into long-term and short-term zones of deafferentation in the LGN in the same experiment. If there was axonal growth corresponding to the spread of excitation, the band of label seen in frontal sections of the LGN should be approximately double the width in the region of long-term deafferentation. No change of this magnitude was observed (Fig. 1d). In contrast, the width of the labelled regions was slightly less in the long-term deafferented parts of the LGN (Table 1) due to the continuous decrease of the retino-geniculate magnification factor towards the periphery of the retina. At this stage our electrophysiological demonstration of lesion-induced plasticity in the adult cat LGN¹ seemed to be at clear variance with all anatomical results^{2–5}.

In an attempt to resolve this discrepancy, we investigated the position of the light-excitable cells relative to the distribution of the input fibres. In two cats a long-term lesion (90 and 97 days) in the upper nasal retina was extended 6 days before the final experiments by a second lesion in the lower nasal retina

Fig. 1 The physiologically observed expansion of light-evoked excitation in the LGN (a,b) is not accompanied by spreading of the retino-geniculate axons (d) originating from a narrow strip of retina bordered by photocoagulator lesions (c). a, Schematic drawing of a frontal section through the cat LGN approximately at the projection of the horizontal meridian of the visual field. Projection columns are indicated for 1°, 5°, 20° and 24° of horizontal eccentricity. Between 1 and 20 days after a nasal retinal lesion at 20° in the contralateral eye the black area shows the deafferented part of layer A. The C laminae (stippled) are not included in the present studies. The 20° projection column is normal, the 24° column ends at the A/A₁ border. b, More than 25 days after deafferentation. Conventions as in a. The 20° projection column has expanded in layer A (hatched region) and now includes the region up to the area previously occupied by the 24° column. Layer A₁ remained unchanged. c, Fundus photograph showing long (upper retina) and short (lower retina) survival-time photocoagulator lesions leaving a strip of normal retina with blood vessels and the papilla (below) unaffected. The orientation of the large vessels is tilted by 20° to the left, relative to the vertical meridian. Scale bar, 10° of visual angle. d, Dark-field photomicrographs from frontal sections of the LGN with bands of autoradiographic label in layers A, C, C₂ and sparing of layers A₁ and C₁. The upper photograph shows a section from the (posterior) short-term deafferented part of the LGN, the lower is from the (anterior) long-term deafferented part. The distance between the sections within the LGN was 96 μ m. Scale bars, 200 μ m.

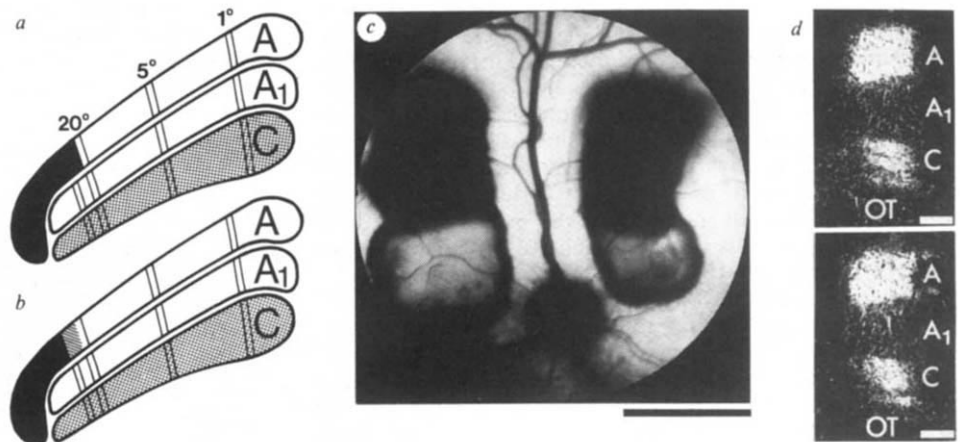
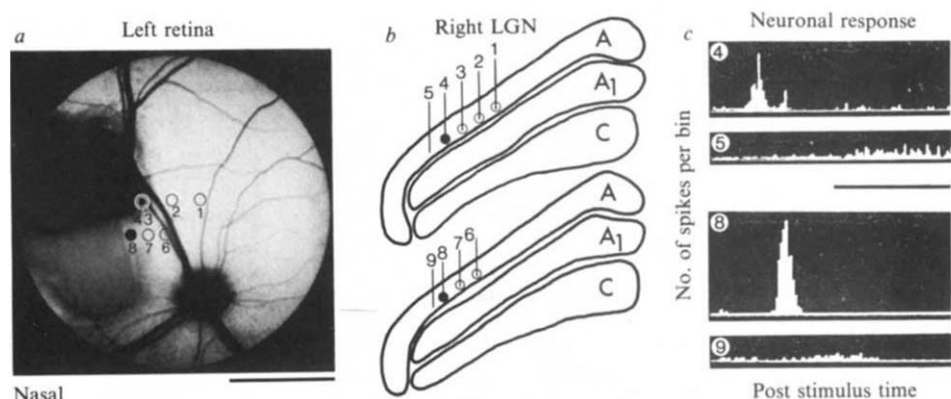


Fig. 2 Receptive field positions (*a*) of neurones, recorded in the LGN (*b*), and responses to flashing lights (*c*) from regions with short- and long-term deafferentation. *a*, Fundus photograph showing the long-term lesion (black) above the short-term lesion (grey). Receptive field positions are marked with numbers. Scale bar, 10° of visual angle. *b*, Line drawings of frontal sections through the contralateral LGN with series of numbered penetrations schematically indicated. Up is dorsal, right is medial. Above, the anterior series of penetrations with correspondingly numbered receptive fields in the upper retina. Below, the series of more posterior penetrations near to the zone of short-term deafferentation. Circles indicate detection of light-excitable cells, black dots symbolize electrolytic lesions at the recording sites of the light-excitable cells closest to the deafferented zones. Interpenetration distances were $150\text{--}200\text{ }\mu\text{m}$, except for $100\text{ }\mu\text{m}$ between 8 and 9. *c*, Neuronal responses to flashing lights. ($n = 64$ flashes, repetition rate 4 s^{-1} .) The numbers indicate the penetrations during which the recordings were made. The responses obtained at the positions of electrolytic lesions close to the regions of long-term (4) and short-term (8) visual deafferentation are paired with typical responses of cells in the nearby unresponsive regions. Time calibration, 50 ms; response calibration, 20 spikes per bin.



of the same eye (Fig. 2*a*). The final experiments were made with anaesthetized and immobilized animals. Single cells were recorded with varnished tungsten microelectrodes¹¹ in layer A of the LGN contralateral to the retinal lesions. The receptive field centres of light-excitable LGN cells were drawn on a map together with the back-projected map of the retina with typical landmarks (vessels, papilla) and both the long- and short-term retinal lesions. The receptive field positions were later transferred to fundus photographs of the lesioned eye (Fig. 2*a*). One series of penetrations of the LGN was made to show the light-excitable cells closest to the LGN region with long-term deafferentation caused by destruction of the upper nasal retina. A second, more caudal series of penetrations was aimed to demonstrate the border of light-excitability next to short-term deafferentation in the LGN due to the lower lesion on the retina (Fig. 2*b*).

The region of long-term geniculate deafferentation contained cells with displaced receptive fields¹ located at the border of the long-established lesions (penetration 4 in Fig. 2*b* yielded the same receptive field position as penetration 3, see Fig. 2*a*). An electrolytic lesion ($0.5\text{--}1.0\text{ }\mu\text{A}$, 10 s, electrode tip negative) was placed to mark this recording site. The maintained activities and the visual responses of the cells with displaced receptive fields were reduced with respect to those recorded from cells with normal retinotopy¹, but the visual responses could be clearly distinguished from the activity obtained in the visually unresponsive regions (Fig. 2*c*). At the border of the short-term lesions no displaced receptive fields could be found and light-excitability ended when the normal projection of the retinal ganglion cells at the border of the retinal lesion was reached in the LGN (penetration 8 in Fig. 2*b*). An electrolytic lesion was also used to mark this geniculate location. The crucial

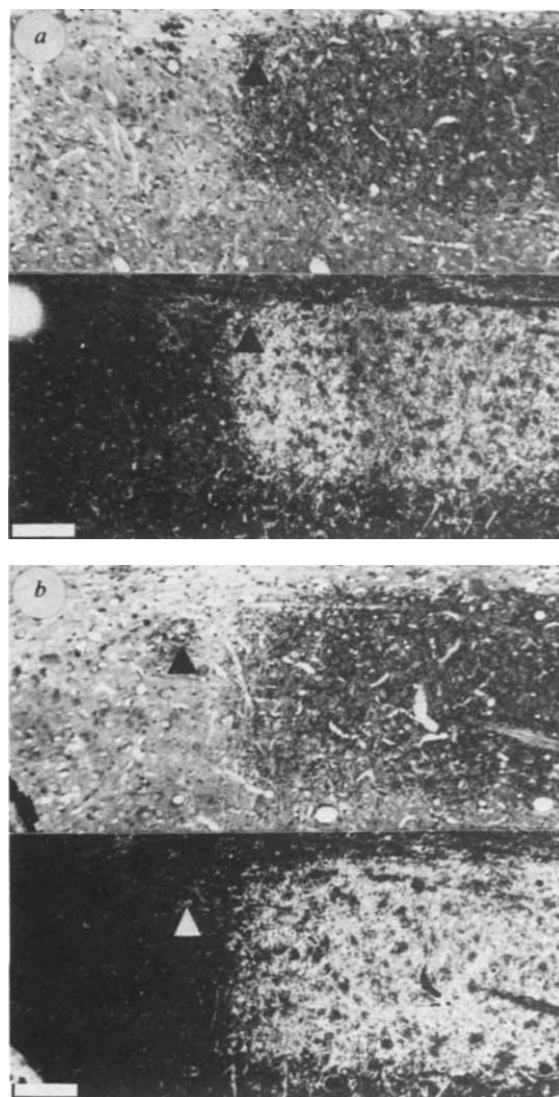


Fig. 3 Autoradiographs of frontal LGN sections with electrolytic lesions at the positions of the light-excitable cells recorded closest to zones of short-term (*a*) and long-term (*b*) deafferentation. *a*, Bright-field and dark-field photomicrographs of layer A. Scale bar, $200\text{ }\mu\text{m}$. The arrowheads indicate the electrolytic lesion within the labelled region. The lesions were clearly defined in the stained sections by a core of damaged tissue surrounded by gliosis. A second electrode track without electrolytic lesion is visible in the unexcitable deafferented region. *b*, Section anterior to the one in *a*. The electrolytic lesion (arrowheads) is in the deafferented, unlabelled zone. Scale bar, $200\text{ }\mu\text{m}$.

Table 1 Medirolateral width of the labelled zones in layer A of the LGN between recently deafferented and long-term deafferented regions

Animal	Short-term deafferentation		Long-term deafferentation	
	Width (μm)	s.d. (μm)	Width (μm)	s.d. (μm)
1	372.31	13.30	335.31	11.45
2	388.50	33.09	352.66	22.88
3*	507.59	22.37	326.06	21.22
4	538.81	18.96	511.06	20.12
5	494.88	17.22	389.66	15.80

In each animal measurements were made from 32 adjacent $6\text{-}\mu\text{m}$ frontal sections. Mean values and standard deviations were separately computed for the 16 sections in the short-term and the 16 sections in the long-term deafferented region.

*In this animal the abnormal reduction of width in the long-term deafferented region was caused by damage of the corresponding part of the retina.

difference between the long- and short-term deafferentation then was that there was excitation extending lateral to the normal projection of the border of the retinal lesion in the LGN only in the long-term case¹ (see Fig. 1b).

Thereafter, 1 mCi of ³H-proline in 30 µl saline was injected into the vitreous body of the experimental eye. The animals survived 24 h and were fixed by vascular perfusion with 4% phosphate-buffered paraformaldehyde (pH 7.2). Serial frontal sections (6 µm) were made through the entire paraffin-embedded LGN, processed for autoradiography (Ilford K2 emulsion, 4–6 weeks' exposure time) and counterstained with cresyl violet through the emulsion. The distribution of radioactive label relative to the electrode tracks with electrolytic lesions was compared in the anterior region with penetrations (long-term deafferentation) and the posterior region with penetrations (short-term deafferentation). After short-term deafferentation the light-excitable geniculate cells were confined to the region of autoradiographically labelled retino-geniculate axons (Fig. 3a). After long-term deafferentation, however, light-excitable cells were found in the deafferented, unlabelled zone as much as 150 µm away from the labelled afferents (Fig. 3b). The second animal which had been subjected to the same experimental procedure yielded practically identical results (data not shown).

This result is interpreted as a demonstration of functional reconnection of formerly deafferented cells by a mechanism not involving significant growth of the retino-geniculate axons. The dendrites of cells in the LGN could provide a structural basis for the described lateral excitation without necessarily undergoing additional growth. Our own observations indicate that the cells with displaced receptive fields after long-term deafferentation received fibres of the fast-conducting (Y) type according to stimulus response latency criteria, after electrical stimulation near the optic chiasm, and thus are most probably Y cells¹². These cells have dendrites with comparatively large lateral extent¹³. A mechanism—probably acting at the periphery of the LGN cell dendrites—may have elevated the excitatory power of visual inputs at formerly ineffective synapses from sub- to supra-threshold levels. Some physiological^{14,15} and behavioural^{8,9} observations have led to the postulation of such post-lesion phenomena in other parts of the mature subcortical central nervous system at borders between regions of deafferentation and surviving afferents. Although collateral axonal sprouting is one of the most discussed mechanisms, sprouting has not been found in many sensory relay nuclei^{2–6,16–18}. Other possible explanations for the changed excitability of deafferented cells are denervation supersensitivity¹⁹, localized formation of new synaptic junctions without significant axonal growth as observed in the septum nuclei of the rat²⁰, translocation of synapses²¹ over distances below the resolution of the autoradiographic method, or dendritic growth²² into the domain of surviving afferents. The present results show that for the LGN of the adult cat absence of significant axonal extension does not imply an absence of functional reorganization, and that alternative mechanisms must be considered.

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Localization of β -adrenoreceptors in mammalian lung by light microscopic autoradiography

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β -Adrenergic agonists regulate a variety of lung functions including relaxation of airway smooth muscle, secretion of mucus¹, release of surfactant² and modulation of mediator release from mast cells³. Assays of the direct binding of radio-labelled β -adrenergic antagonists to homogenates of whole lung from several species have revealed a very high density of β -adrenoreceptors, but the cellular location of these receptors has not been determined^{4–7}. Using a technique previously used to study distribution of receptors in the brain^{8,9} we report here for the first time the localization of β -receptors in the lung by light microscopic autoradiography. Initial biochemical studies showed that ³H-dihydroalprenolol (³H-DHA) bound to slide-mounted frozen sections of ferret lung with the characteristics expected of interaction with β -receptors. Autoradiograms prepared by apposition of dry emulsion-coated coverslips to these sections showed that the distribution of β -receptors in the lung was widespread. The highest concentration of β -receptors was found in smooth muscle of bronchioles with somewhat lower densities in smooth muscle of large airways. Labelling also occurred in airway epithelium, submucosal glands and vascular smooth muscle. Alveolar walls were heavily labelled and as type II pneumocytes, which are known to have β -receptors, comprise only a small portion of the surface area, β -receptors must also be present on type I cells and/or capillary endothelial cells, although their function is at present unknown.

The lung contains over 40 different cell types and, although radioligand binding assays show the density of β -receptors in whole lung homogenates to be higher than in any other tissue^{4–7}, the localization of β -receptors in the lung has not been possible by assay alone. Previous attempts to study distribution of β -receptors in tissues, including lung, using a fluorescent β -antagonist¹⁰ were unsuccessful, largely because of the low affinity of the fluorescent analogue¹¹. Another method using ³H-DHA given by intravenous injection showed *in vivo* labelling of pulmonary β -receptors, but the ratio of specific to nonspecific binding was low, and interpretation confounded by *in vivo* metabolism, unequal distribution of the radioligand and competition for binding sites by endogenous catecholamines¹². These problems do not arise with slide-mounted sections of lung and detailed biochemical studies of such sections showed that ³H-DHA bound to frozen sections of ferret lung with all the characteristics expected of interactions with β -receptors. Specific binding was defined as that prevented by 1 µM (–)propanolol and accounted for 80–90% of total binding at concentrations of ³H-DHA below 2 nM. Specific binding was saturable and Scatchard analysis showed a single class of binding sites with a maximum binding capacity (B_{max}) of 16.2 ± 3.2 fmol

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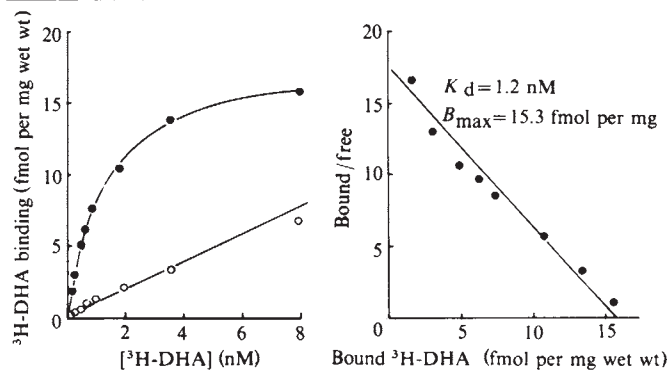


Fig. 1 Equilibrium binding of $^3\text{H-DHA}$ to frozen sections of ferret lung. *a*, Saturation curve showing specific (●) and non-specific (○) binding in the presence of $1 \mu\text{M}$ (–)propranolol. *b*, Scatchard plot showing a single class of binding site. The inverse of the slope determined by linear regression ($r = 0.96$) gives a K_d of 1.2 nM and its intercept with the abscissa shows a B_{max} of $15.3 \text{ fmol per mg wet weight}$ of tissue. Each point is the mean of triplicate determinations in one experiment. Separate determinations in other animals gave a K_d of $1.4 \pm 0.27 \text{ nM}$ (mean $\pm$ s.e.m., $n = 4$) with B_{max} of $16.2 \pm 3.2 \text{ fmol per mg wet weight}$. Ferrets (*Mustela putorius*) were anaesthetized with pentobarbitone (35 mg kg^{-1} , intraperitoneally) and the lungs rapidly removed. Individual lobes were cannulated and inflated with saline-diluted OCT embedding fluid (Lab-Tek) then rapidly frozen in freon cooled by liquid nitrogen. Cryostat sections ($8\text{--}16 \mu\text{m}$ thick) were cut at -15°C and thaw-mounted onto gelatinized glass microscope slides. After washing to remove OCT, slides bearing 3 to 4 sections were incubated with 0.1 to 8 nM $^3\text{H-DHA}$ (specific activity 101 Ci mol^{-1} NEN) in 50 mM Tris-HCl buffer, $\text{pH } 7.4$ at 25°C for 20 min . Alternate lung sections were incubated with $^3\text{H-DHA}$ in the presence of $1 \mu\text{M}$ (–)propranolol to determine nonspecific binding. Slides were washed in buffer at 4°C to remove unbound radioligand. Washing for 10 min gave the greatest specific:nonspecific ratio with a decrease in nonspecific radioactivity of over 90% , but a decrease in specific radioactivity of only 40% . Sections from each slide were then scraped into pre-weighed glass scintillation vials and the wet weight of tissue determined, (approximately 10 mg per vial). After incubation with tissue solubilizer (Protosol, NEN) at 60°C for 6 h , vials were counted in a Beckman liquid scintillation spectrometer at an efficiency of 50% . Specific binding, calculated from the difference in counts between total and non-specific binding, was $80\text{--}90\%$ of total counts bound.

per mg wet weight of tissue (mean $\pm$ s.e.m., $n = 4$, Fig. 1). Binding was of high affinity with a K_d of $1.4 \pm 0.27 \text{ nM}$. This was not significantly different from the binding affinity measured in a washed membrane preparation of whole lung from these animals ($K_d = 1.3 \pm 0.15 \text{ nM}$, $n = 5$) determined as previously described¹³, indicating that preparation of the lung sections did not alter binding characteristics. Specific binding was stereoselective with (–)propranolol ($K_i = 1.6 \text{ nM}$) showing over 100 times greater potency than propranolol in inhibiting specific binding (Fig. 2). Amongst agonists, isoprenaline ($K_i = 0.09 \mu\text{M}$) was more potent than adrenaline ($K_i = 0.4 \mu\text{M}$), with noradrenaline ($K_i = 5.2 \mu\text{M}$) having only a weak effect. Pulmonary β -receptors, therefore, seem to be predominantly of the β_2 -subtype. Phentolamine and atropine had no significant inhibitory effect on binding. Specific binding was rapid and had reached equilibrium by 10 min . Washing at 4°C for 10 min was found to reduce nonspecific binding and provided the optimal specific: nonspecific ratio.

Autoradiography revealed that β -receptors were widely distributed throughout the lung. Labelling was confined to cellular regions with no significant elevation of radioactivity above background levels in interstitial spaces, signifying that no diffusion of the radioligand had occurred during processing (Figs 3, 4). Adjacent sections which had been incubated with $^3\text{H-DHA}$ in the presence of $1 \mu\text{M}$ (–)propranolol showed relatively few autoradiographic grains that did not coincide with tissue structures in a specific pattern. There was dense labelling of airway smooth muscle and this was greater in small airways

(bronchioles) than in large airways (cartilaginous bronchi). The presence of β -receptors on airway smooth muscle is not surprising as β -agonists are potent bronchodilators. Our study shows that they are likely to cause dilation of all airways because of the widespread distribution of β -receptors in muscle of airways of all sizes. β -Agonists stimulate ion transport and fluid secretion in tracheal epithelium¹⁴ and $^3\text{H-DHA}$ binds specifically to isolated tracheal epithelial cells (P.J.B. and J. H. Widdicombe, unpublished observations). The finding of β -receptors on epithelium of all sizes of airway suggests that β -receptors may regulate fluid movement in the intrapulmonary airways as well. This is supported by the demonstration that β -agonists increase mucociliary clearance by the lung¹⁵. β -Receptors were also found in submucosal glands of large airways, which is consistent with the finding that β -agonists stimulate the secretion of airway mucus. There is increasing evidence that β -agonists stimulate secretion primarily from mucous rather than serous cells of airway glands¹⁶, but it was not possible to distinguish these cell types in our cryostat sections. There was almost no specific labelling in connective tissue and it was not possible to identify mast cells. Vascular smooth muscle showed some labelling, consistent with the finding that β -agonists cause relaxation of pulmonary arteries¹⁷, but there was no labelling of endothelial cells of large vessels.

The most surprising finding was the uniformly dense labelling of alveoli. β -Agonists stimulate release of surfactant from alveolar type II pneumocytes² and radioligand binding to isolated type II cells has demonstrated the presence of β -receptors¹⁸. However, type II cells line less than 3% of the alveolar surface, with the alveolar wall being comprised predominantly of type I pneumocytes and capillary endothelial cells¹⁹. The role of β -receptors on type I cells or endothelial cells has not been studied. It is possible that β -receptors may regulate fluid resorption from the alveolar spaces, particularly at birth, as catecholamines are known to reduce fetal alveolar fluid content²⁰. Alternatively, β -receptors on type I cells may have no physiological role but may be present on type I cells as a vestige of their derivation from type II cells. In any case the large number of β -receptors in the alveolar walls explains the very high density of β -receptors found in membrane preparations

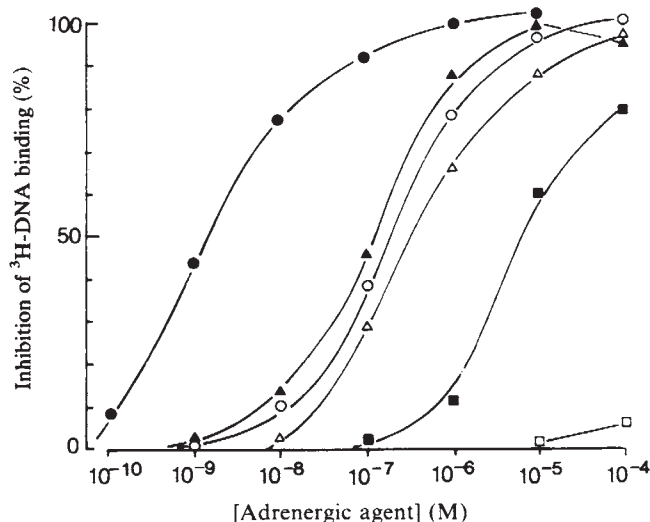


Fig. 2 Inhibition of specific $^3\text{H-DHA}$ binding to ferret lung sections by increasing concentration of adrenergic agents. Each point is the mean of three determinations which did not differ by more than 10% . Assays were performed as described in Fig. 1 using 1.5 nM $^3\text{H-DHA}$. The concentration of drug causing 50% inhibition of binding (IC_{50}) was determined, and the inhibitory dissociation constant (K_i) was calculated from the formula $K_i = \text{IC}_{50} / [1 + K_d / (L)]$ where K_d is the dissociation constant of $^3\text{H-DHA}$ binding and (L) is the concentration of $^3\text{H-DHA}$ used in the assay. ●, (–)Propranolol; ▲, (–)isoprenaline; ●, (+)propranolol; Δ, (–)adrenaline; ■, (–)noradrenaline; □, phentolamine.

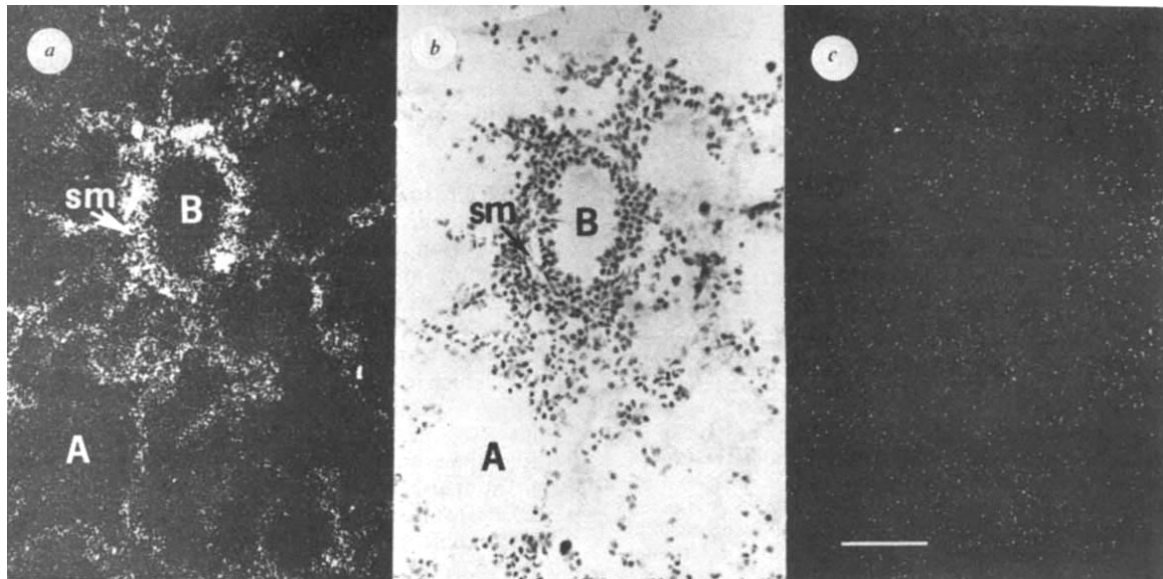


Fig. 3 Distribution of β -receptors in peripheral lung of ferret. *a*, Dark-field photomicrograph of the section shown in *b*, stained with cresyl violet. The section shows a small bronchiole (B) with surrounding alveoli (A). Note the dense labelling of patches of smooth muscle (sm) in the bronchiolar walls and the uniform labelling of alveolar wall. Scale bar, 100 μ m. *c*, Dark-field photomicrograph of an adjacent section, incubated in an identical way but in the presence of 1 μ M (-)propranolol, shows absence of specific labelling. Cryostat sections 8 μ m thick were incubated with 1.5 nM 3 H-DHA at 25 $^{\circ}$ C for 20 min as described in Fig. 1. After washing, sections were rapidly dried in a stream of cold air then left to dry overnight at 4 $^{\circ}$ C. Glass coverslips, which had been coated with photographic emulsion (Kodak NTB 2 diluted 1:1 with water) and dried overnight, were then fixed to the slides at the end distant from the section with cyanoacrylate glue and closely applied to the section with binder clips. Slides were stored in light-proof boxes with desiccant capsules at 4 $^{\circ}$ C for 12 weeks. The coverslips were then removed using a diamond pencil to score and break the glass distal to the glue and developed (Kodak D19 for 3 min at 17 $^{\circ}$ C). The sections were stained with cresyl violet and dried coverslips reapposed over the tissue using scored guide-lines to reposition exactly. Slides were then examined with a Zeiss microscope equipped for bright-field and dark-field illumination. Grain counts were performed under bright-field illumination using a $\times 40$ objective lens and a calibrated eyepiece grid. Grain densities were expressed as autoradiographic grains per 1,000 μ m² (mean $\pm$ s.e.m.) and were determined from 10 separate fields in 3 different sections. To obtain specific grain densities, nonspecific grain densities in the presence of 1 μ M (-)propranolol (29–39) were subtracted for total grain densities. Specific grain counts: bronchiolar smooth muscle 358 $\pm$ 18, large airway smooth muscle 185 $\pm$ 17, airway epithelium 74 $\pm$ 5, airway gland 109 $\pm$ 6, alveolar wall 263 $\pm$ 16, vascular smooth muscle 78 $\pm$ 6, airway connective tissue 8 $\pm$ 2. A similar pattern of distribution was seen in four separate animals.

from whole lung homogenates as most of the cell membranes of the lung are derived from alveolar cells.

There is considerable variation between species in sympathetic innervation of the lungs²¹. In many species there is an adrenergic nerve supply to blood vessels and airway glands but not to smooth muscle of intrapulmonary airways. Using a fluorescent histochemical technique²² to study distribution of

adrenergic nerve fibres in cryostat sections of ferret lung we found sympathetic innervation of vascular smooth muscle as expected, but also of smooth muscle of intrapulmonary cartilaginous airways. There was no detectable innervation of bronchioles or alveolar walls, the structures having the highest density of β -receptors, indicating that these receptors are probably controlled by circulating catecholamines.

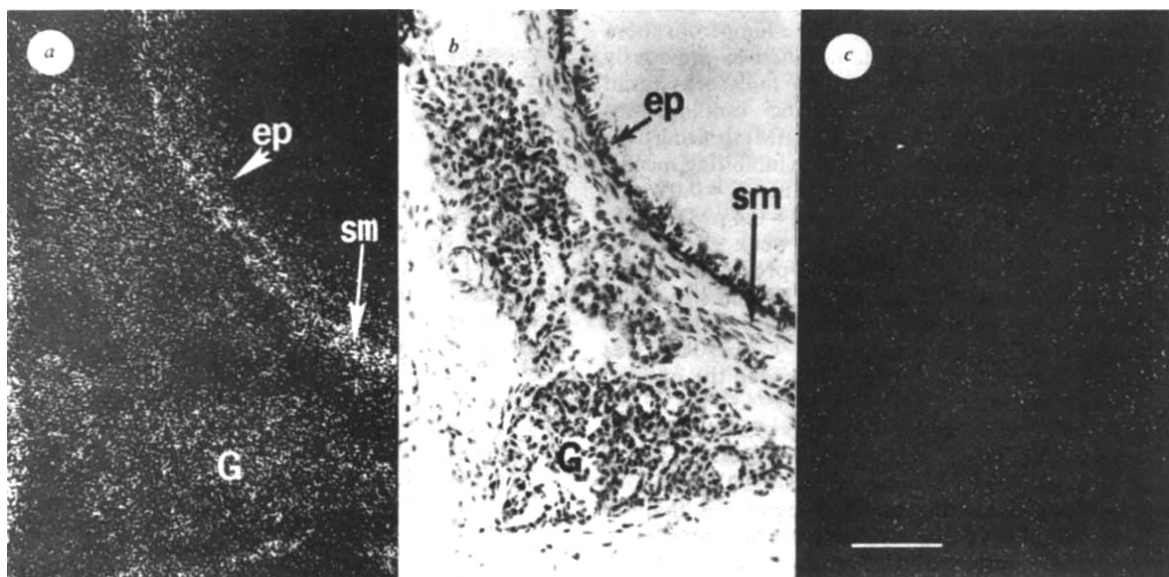


Fig. 4 Distribution of β -receptors in a large airway (segmental bronchus) of ferret. *a*, Dark-field photomicrograph of sections shown in *b*. Note the dense labelling of airway smooth muscle (sm) with lighter labelling of epithelium (ep) and submucosal glands (G). Scale bar, 100 μ m. *c*, The same area in an adjacent section incubated in the presence of 1 μ M (-)propranolol with no specific labelling.

In homogenates of whole lung, changes in the density of β -receptors have been shown during development^{23,24}, with steroid treatment^{23,25} and in an animal model of asthma⁶. It may now be possible to study the cellular location of these changes in pulmonary β -receptor density, leading to further advances in our understanding of the role of β -receptors in the regulation of various lung functions.

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Structural and biological properties of a monoclonal auto-anti-(anti-idiotypic) antibody

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Several studies illustrating idiosyncrasy network-directed regulation of the immune response¹ have demonstrated that heterologous anti-(anti-idiotypic) antibodies (1) share variable (V) region antigenic determinants with idiotypic-bearing antibodies^{2–5}, (2) lack antigen-binding activity^{3–6} and (3) alter the clonal profile of an immune response by stimulating/suppressing regulatory lymphocyte clones of anti-idiotypic specificity^{2–4,6}. However, the existence and significance of anti-(anti-idiotypic) antibody in natural immunological responses has been questioned because of the artificial immunization procedures used to induce these antibodies. Here we describe a monoclonal mouse anti-(anti-idiotypic) antibody generated in physiological conditions by immunizing BALB/c mice with a pneumococcal vaccine that elicits a humoral anti-phosphorylcholine (PC) response dominated by the T15 idiosyncrasy⁷. This antibody recognized an idiotope on a monoclonal anti-T15 antibody, failed to bind PC, did not exhibit detectable idiosyncrasy cross-reactivity with T15⁺ immunoglobulins, utilized V_H and V_L genes distinct from the T15 V gene group⁸, and exerted a suppressive effect on the *in vivo* response to PC in syngeneic mice.

Spleen cells were taken from BALB/c mice 4 days after intravenous (i.v.) immunization with heat-killed *Streptococcus pneumoniae* strain R36A and were fused with the non-secreting myeloma P3×63Ag8.653 (ref. 9). In initial screening of the

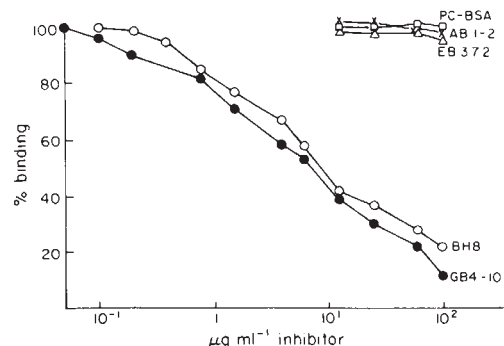


Fig. 1 Determination of binding specificity for MM-60. Various monoclonal antibodies and PC-bovine serum albumin (PC-BSA) were tested for ability to inhibit reactivity between immobilized MM-60 and alkaline phosphatase-conjugated GB4-10 in an enzyme-linked solid phase immunosorbent assay (ELISA)¹⁰. *p*-Nitrophenylphosphate (1 mg ml⁻¹) was used as substrate and reactivity was quantitated using absorbance measurements at 405 nm. Wells containing incubation buffer served as control samples (100% binding). PC-BSA was synthesized by the method of Chesebro and Metzgar²⁴ and immunoglobulins were purified by ion exchange or affinity chromatography. Inhibition values for DB1-1, B24-44, PC12-3, FD5-3 and affinity-purified goat anti-T15 antibody (not shown) are virtually identical to those found for AB1-2 (97–100% binding at 100 μg ml⁻¹ inhibitor). Recognition of PC hapten by MM-60 could not be determined using this assay since binding of GB4-10 to T15⁺ antibody is not inhibited by PC hapten¹⁰. AB1-2, DB1-1 and B24-44 are distinct anti-T15 idiosyncrasy antibodies^{10,29}; BH8 is an IgMκ AB1-2⁺, GB4-10⁺ anti-PC antibody; PC12-3 is an IgMκ AB1-2⁺, GB4-10⁻ anti-PC antibody; EB3.7.2 is J558 (α1→3 dextran-binding myeloma) idiotype-specific; and FD5-3 recognizes an idiotope on MOPC 460 (dinitrophenol-binding myeloma).

hybridoma supernatants, one hybridoma (MM-60) produced an antibody (IgMλ) that reacted with the monoclonal A/J anti-T15 idiosyncrasy antibody GB4-10 (ref. 10), but did not bind to PC. After subsequent cloning, the specificity of purified MM-60 was tested using a battery of monoclonal BALB/c and A/J antibodies in a solid-phase immunosorbent binding inhibition assay. As shown in Fig. 1, MM-60 was specific for GB4-10 and did not recognize allotypic or isotypic determinants (irrelevant anti-idiosyncrasy hybridoma proteins FD5-3 and EB3.7.2 are of identical allotype and isotype). BH8, an anti-PC hybridoma protein reactive with GB4-10, effectively inhibited binding of MM-60 and GB4-10, providing additional evidence that the recognition of idiosyncrasy determinants was involved. General idiosyncrasy cross-reactivity between MM-60 and T15⁺ immunoglobulin was not observed, as other monoclonal anti-T15 idiosyncrasy antibodies and purified heterologous goat anti-T15 idiosyncrasy antibody did not inhibit the MM-60/GB4-10 interaction (Fig. 1).

Several observations suggested that MM-60 was anti-(anti-idiosyncrasy), rather than an immunoglobulin that merely expressed the GB4-10-defined idiotope. First, MM-60 did not bind to PC-protein conjugates (Fig. 1 and in direct binding assays), in contrast to all GB4-10-bearing proteins identified so far^{10,11}. Second, MM-60 did not express the heavy chain-associated idiotope DB1-1 (Fig. 1), a characteristic of all GB4-10-bearing immunoglobulins (J.K., unpublished). In addition, it has been demonstrated that the binding of GB4-10 to murine splenic lymphocytes was completely abolished in the presence of soluble PC-protein conjugates (E. J. B. Bast and J.K., unpublished). The apparent restriction in expression of the GB4-10 idiotope among immunoglobulins to the T15⁺ anti-PC group strongly argues that MM-60 is not expressing a 'public' idiotope (that is, GB4-10) on unrelated V regions of different antigen specificity.

Somatic variants of a T15⁺ anti-PC myeloma have been isolated that have lost binding activity for PC-protein conjugates^{12,13}; presumably MM-60 could be such a variant that has

Fig. 2 Comparison of N-terminal V_H sequence for MM-60 and TEPC 15. MM-60 heavy chain was isolated by molecular sieve chromatography after exhaustive reduction and alkylation of the IgM fraction from MM-60 ascites fluid²⁵. Amino acid sequencing was performed by sequential Edman degradation on a modified Beckman 890C automated sequencer²⁶. The TEPC 15 sequence is from Barstad *et al.*²⁷. Sequence differences between MM-60 and TEPC 15 V_H are underlined, invariant residues (for murine heavy chain group III²⁸) are italicized and residues tentatively identified are shown in parentheses.

MM-60	Asx-Val-Gln-Leu-Val-Glu-Ser-Gly-Gly-Gly-Leu-Val-(Gln)-Pro-Gly-Gly-Ser-(Leu)-Lys-Leu-
TEPC 15	Gly-Val-Lys-Leu-Val-Glu-Ser-Gly-Gly-Gly-Leu-Val-Gln-Pro-Gly-Gly-Ser-Leu-Arg-Leu-
	1 5 10 15 20

also lost several T15-associated idiotopes, while retaining the GB4-10 idiotope. MM-60 and T15⁺ immunoglobulins would then utilize identical germ-line V_H and/or V_L genes. However, different V_L genes are used as MM-60 contained λ light chains and all anti-PC antibodies have κ light chains¹⁴. Comparison of the N-terminal heavy chain sequence for MM-60 and the prototype antibody TEPC 15 (Fig. 2) showed at least three sequence differences among framework residues 1 through 20, demonstrating that these two antibodies belong to distinct V_H groups¹⁵. Within this region of the V_H domain, T15⁺ antibodies and their characterized antigen-binding variants exhibit 100% homology¹⁶.

In conjunction with its unique chemical properties, MM-60 was capable of suppressing the *in vivo* response to PC in an idiotype-specific manner (Table 1). CAF₁ (BALB/c $\times$ A/J) mice treated with the anti-(anti-idiotypic) and subsequently challenged with R36A vaccine produce serum anti-PC IgM at 20% of control levels; the T15 component of the response was preferentially affected with only 8% of the normal serum levels of T15⁺ IgM made. MM-60-induced suppression was specific for the PC response (Table 1), was attained using low doses of antibody (variable degrees of suppression were accomplished in a dosage range of 10 ng–100 μ g), and was chronic only for the T15 component of the response (78% suppression in T15⁺ antibody production at 10 weeks after anti-(anti-idiotypic) treatment). Furthermore, a similar pattern of suppression can be effected in BALB/c mice (not shown).

Lymphocytes that express anti-T15 receptors containing the GB4-10 idiotope and that specifically regulate T15⁺ B cells are the probable targets for MM-60 *in vivo*. Several cell populations that could satisfy these requirements have been described; anti-T15 idiotypic B cells¹⁷, suppressor T cells¹⁸ and helper T cells¹⁹. Using a Western blot analysis²⁰ (Fig. 3), we found that MM-60 recognized a determinant on GB4-10 that is dependent

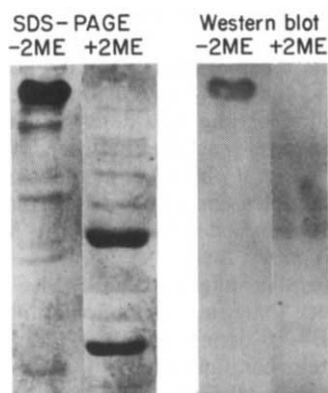


Fig. 3 Expression of the MM-60-defined idiotope requires associated heavy and light chains. SDS-polyacrylamide gel electrophoresis²⁵ was performed on the GB4-10 protein ($\gamma_1\kappa$, equal amounts) in reducing (5% 2-mercaptoethanol) and non-reducing conditions. The Western blotting technique described by Towbin *et al.*²⁰ was followed; electrophoretic transfer of the protein from slab gel to nitrocellulose paper was carried out for 3 h and unreacted sites on the paper were blocked with a 3% BSA (w/v) and 5% carrier mouse serum (v/v) solution in borate-buffered saline. ¹²⁵I-MM-60 (10^5 c.p.m. ml⁻¹) was incubated with the nitrocellulose sheet for 3 h at 37°C with gentle swirling. After extensive washing, autoradiography of the paper was performed at -70°C for 30 h. MM-60 reacted only with the intact GB4-10 molecule and no reaction of MM-60 at heavy or light chain bands was observed on increasing exposure time (4 days).

Table 1 MM-60-induced suppression of anti-PC and T15⁺ IgM response

Assay	Serum IgM (μ g ml ⁻¹ $\pm$ s.e.)	
	MM-60-treated	Control
Anti-PC	4.6 $\pm$ 1.4	19.8 $\pm$ 2.7
T15 ⁺		
(a) AB1-2 ⁺	1.3 $\pm$ 1.3	16.8 $\pm$ 3.0
(b) GB4-10 ⁺	1.0 $\pm$ 1.1	17.3 $\pm$ 3.3
Anti- α 1 $\rightarrow$ 3 dextran ^a	424 $\pm$ 10.1	392 $\pm$ 11.2

Two groups of five 8-week-old CAF₁ female mice [(BALB/c $\times$ A/J)F₁] received either two intraperitoneal (i.p.) injections of 100 μ g MM-60 antibody (purified from tissue culture supernatant by affinity chromatography on a goat anti-mouse column) or two injections of saline (5 days interval). All mice were challenged (i.p.) 3 days later with 2×10^8 heat-killed *S. pneumoniae* strain R36A and bled 5 days afterwards. Serum antibody was quantitated for anti-PC and T15⁺ IgM using an enzyme-linked immunosorbent direct binding assay¹⁰ (AB1-2 and GB4-10 are idiotope markers for T15 expression); linear regression analysis of binding activity over twofold serum dilutions was carried out. Absence of nonspecific immune suppression caused by MM-60 treatment was shown by measuring the response to α 1 $\rightarrow$ 3 dextran (Dex) in similarly suppressed CAF₁ mice^a (10 μ g i.p.; Dex and R36A vaccine are classified as TI-2 antigens²³). Mice were bled 7 days later and serum antibody quantitated as before (Dex-BSA used as immobilized antigen for the assay). Statistical comparison of experimental versus control groups using a two-sample rank test gave *P* values < 0.02 for anti-PC and T15⁺ assays.

on associated heavy and light chains for its expression. Thus it seems likely that the reactivity of our monoclonal anti-(anti-idiotypic) antibody is limited to anti-idiotypic B cells, as the T-cell antigen receptor apparently lacks light chains²¹ and seems to express only V_H -associated idiotopes²². Indirect fluorescent antibody staining of the GB4-10 hybridoma cell surface confirmed that MM-60 can recognize its respective determinant on membrane-bound immunoglobulin.

Although MM-60 exerted a dampening influence on the appropriate B-cell repertoire, heterogeneous anti-(anti-idiotypic) antibodies in other antigen systems generally potentiate the corresponding humoral response to antigen. This discrepancy has several possible explanations: (1) Many of the autologous anti-(anti-idiotypic) antibodies previously studied correspond to silent idiotypic systems instead of dominant responses like PC/T15. (2) Anti-(anti-idiotypic) antibody generated under intensive immunization regimens might not reflect those anti-(anti-idiotypic) clones which are stimulated during the course of a natural immune response (as to specificity or isotype). And (3) heterologous anti-(anti-idiotypic) antibody preparations can contain idiotype-bearing immunoglobulin⁴ which could possess disparate immunoregulatory activity. However, our results do support the general significance of idiotype network interactions in the regulation of antibody production. Further cloning of lymphocytes that arise at various times during an immune response should help in understanding these reactions.

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Chromatin changes accompany immunoglobulin κ gene activation: a potential control region within the gene

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A functional immunoglobulin gene is formed by the fusion of individual coding elements which are widely separated in germ-line DNA. For the mouse κ light chain gene, this is accomplished by DNA rearrangement which brings one of a large repertoire of non-allelic variable region (V_κ) genes into apposition with one of four junctional (J_κ) elements located 2.7–3.9 kilobases (kb) upstream from the κ constant region coding sequence (C_κ)^{1–4}. Transcription is initiated near the 5' end of the rearranged V_κ locus, and spans all three coding elements and the large intervening sequence between J_κ and C_κ , and a smaller intervening sequence within the V_κ gene⁵. Little is known of the mechanisms which control the expression of rearranged immunoglobulin genes. We have studied the process of κ light chain gene activation in a mouse leukaemia cell line, 70Z/3, which synthesizes a κ -type light chain protein in response to bacterial lipopolysaccharide (LPS). Here we describe changes in the chromatin structure of the κ genes occurring as a result of LPS treatment, and present evidence that a discrete region located inside the large intervening sequence of the κ transcription unit plays a part in κ gene activation.

Cells of the 70Z/3 line exhibit several features typical of the early stages of B lymphocyte differentiation^{6–9}. The cells constitutively produce cytoplasmic μ heavy chains without detectable light chain synthesis. On exposure to LPS, however, the cells initiate synthesis of κ light chain protein, and begin to express both heavy and light chains on their surfaces. The response of 70Z/3 cells to LPS is rapid and uniform. In the absence of LPS, most cells (>90%) have no light chain protein; after optimal LPS treatment, 70–100% express surface and cytoplasmic κ determinants^{6,7}. Because maximal expression can be achieved within 12 h, this phenotypic change probably does not arise through selection of an endogenous subpopulation. The cells carry a single rearranged κ gene; the excluded κ allele retains the germ-line configuration⁸. Despite extensive restriction enzyme mapping of regions in and around the two κ alleles

of 70Z/3, we have detected no changes in the sequence organization of either gene occurring as a result of exposure to LPS. This observation implies that the light chain produced by 70Z/3 is a product of the constitutively rearranged allele. While untreated 70Z/3 cells contain little or no cytoplasmic κ -specific RNA, exposure to LPS results in an accumulation of 50–100 copies of mature-sized κ mRNA per cell⁹, suggesting that LPS induces transcription of the κ gene. More rigorous evidence for an effect of LPS on κ transcription in 70Z/3 will be presented elsewhere¹⁰.

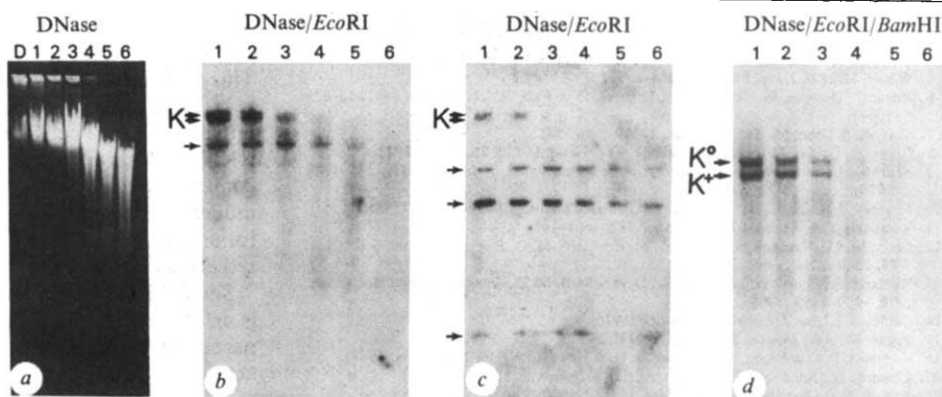
Several lines of evidence suggest that transcriptional activity is an inherent property of the C_κ region, and is imparted to a particular V_κ gene only after rearrangement has linked it to an active C_κ locus. Thus, while each V_κ element in the genome apparently harbours a potential initiation site near its 5' end, unrearranged V_κ genes are not transcribed¹¹, and do not show the enhanced accessibility to nuclease digestion characteristic of transcriptionally active chromatin¹². In contrast, unrearranged C_κ alleles found in myeloma cells are transcribed^{5,13,14}, and are nuclease-sensitive to a degree comparable with that of rearranged C_κ genes¹². It is not known whether transcription is the cause or the result of nuclease sensitivity in these genes.

To characterize the effect of LPS on immunoglobulin gene structure, we first examined the chromatin conformation of the light chain genes of untreated 70Z/3 cells, by determining the susceptibility of these genes to digestion with pancreatic deoxyribonuclease I (DNase I). Nuclei from cells grown in the absence of LPS were subjected to mild digestion with various concentrations of DNase. DNA isolated from these nuclei showed a gradual decrease in mean fragment size with increasing DNase digestion (Fig. 1a). These DNA preparations were then cleaved to completion with selected restriction endonucleases, subjected to agarose gel electrophoresis, and then transferred to nitrocellulose membranes. Specific gene sequences were identified by hybridization to radiolabelled recombinant DNA probes, followed by autoradiography. When undigested 70Z/3 DNA was cleaved with the enzyme *EcoRI* and probed for κ constant region coding sequences (Fig. 1b, lane 1), two bands (labelled K) were observed, corresponding to the germ-line (15 kb) and rearranged (17 kb) C_κ alleles⁸. Both these κ -bearing fragments were relatively sensitive to nuclease action, and disappeared rapidly as nuclei were exposed to increasing concentrations of DNase (Fig. 1b, lanes 2–6). In contrast, the λ I light chain genes, which are neither rearranged nor expressed in these cells, were comparatively resistant to DNase digestion (Fig. 1b, unlabelled arrow). Similar results were obtained for DNA preparations probed simultaneously for C_κ and albumin gene sequences: both κ alleles were significantly more sensitive than the unexpressed albumin sequences (Fig. 1c). Analysis of these same DNA preparations after combined cleavage with *EcoRI* and *BamHI* gave better resolution of the two C_κ -bearing fragments (Fig. 1d) and revealed no difference in nuclease sensitivity between the rearranged (K^+) and germ-line (K^0) alleles.

These findings indicate that the κ constant region genes of untreated 70Z/3 cells exist in a chromatin conformation which renders them preferentially accessible to DNase digestion compared with transcriptionally inactive chromatin, and that this alteration in chromatin structure affects both rearranged and unrearranged C_κ genes. Similar observations have been made for the κ genes of mature B lymphocytes and plasma cells¹²; the present findings suggest that this altered chromatin structure may be established at a very early stage in B-cell ontogeny, and can be maintained in the absence of κ gene expression.

Similar analysis of 70Z/3 cells treated with LPS revealed a striking change in the digestion pattern of κ -specific chromatin. Combined *EcoRI* and *BamHI* cleavage of DNA from LPS-treated cells produced the two expected C_κ -bearing fragments, which were rapidly digested in nuclei exposed to DNase (Fig. 2). During DNase digestion, however, a prominent 2.1-kb subfragment accumulated. This subfragment was generated by mild DNase treatment, and was eventually degraded after

Fig. 1 Preferential accessibility of inactive κ light chain genes to nuclease digestion. 70Z/3 cells were grown in suspension culture as described elsewhere⁹ in the absence of LPS. The absence of detectable κ gene expression was confirmed by surface fluorescence immunoassay and by RNA blot hybridization²⁶. Nuclei were isolated from washed cells by the method of Jackson and Chalkley²⁷, and suspended at a DNA concentration of 1 mg ml^{-1} in ice-cold 0.25 M sucrose, 10 mM Tris pH 8.0, 10 mM MgCl_2 . Aliquots (0.25 ml) of the nuclear suspension were preincubated at 37°C for 1 min. Pancreatic DNase I (Worthington DFP grade) was added to a final concentration of $0.4\text{--}4.0 \text{ U ml}^{-1}$, and incubation continued for 2 min. Digestion was stopped by adding 0.1 vol each of 10% SDS and 2 mg ml^{-1} proteinase K. After incubation at 37°C for 2 h, the reaction mixture was extracted twice with anhydrous ether and adjusted to 0.5 M NaCl. Nucleic acids were precipitated by addition of 2.5 vol ethanol. The precipitate was resuspended in 10 mM Tris pH 8.0, 0.1 mM EDTA, and digested successively with 0.1 mg ml^{-1} ribonuclease A and 0.2 mg ml^{-1} proteinase K for 2 h each at 37°C . The samples were extracted twice with phenol/chloroform (1:1) and twice with anhydrous ether, then adjusted to 0.2 M ammonium acetate and precipitated with ethanol. After digestion with selected restriction enzymes, aliquots of the purified DNA were electrophoresed on 0.6% agarose gels and transferred to nitrocellulose²⁸. Hybridization to radiolabelled probes was carried out for 18 h at 65°C in 0.72 M NaCl, 40 mM sodium phosphate pH 7.0, 4 mM EDTA, and 10% (w/v) dextran sulphate. Filters were washed and autoradiographed as described elsewhere²⁶. Hybridization probes were labelled by nick-translation²⁹ of purified cloned restriction fragments. κ Constant region sequences were detected with a 300-base pair fragment obtained by *HincII-HinfI* digestion of a cloned κ complementary DNA (unpublished). The probe for λI constant region sequences was prepared as described elsewhere³⁰ from a recombinant plasmid supplied by A. Bothwell and D. Baltimore. The probe complementary to rat albumin sequences was a gift from J. Taylor³¹. *a*, Purified DNA before restriction digestion: lane D is unincubated DNA; lanes 1–6 are from nuclei incubated with increasing concentrations of DNase, ranging in equal increments from 0 to 4 U ml^{-1} . *b*, *EcoRI* digests ($10 \mu\text{g}$ DNA per lane), probed simultaneously for κ and λI constant region sequences. *c*, *EcoRI* digests ($10 \mu\text{g}$ DNA per lane), probed for κ constant region sequences. Corresponding lanes in *a*–*d* are from the same DNA preparation, and therefore can be compared directly. A 50% reduction in the image density of the κ fragments occurred at a DNase concentration of $0.8\text{--}1.6 \text{ U ml}^{-1}$; this value was essentially unaffected by a 60% reduction in the size of the fragments (compare *b* and *d*). A 50% reduction in intensity of the λ and albumin fragments required ~ 2.4 and 3.2 U ml^{-1} , respectively. K denotes the κ genes; K° and K^+ , the embryonic and rearranged κ genes, respectively. Unlabelled arrows indicate λI genes in *b* and albumin sequences in *c*.



further digestion. The subfragment was observed in DNA from digested nuclei after cleavage with any of a variety of restriction endonucleases, and varied in size according to the particular restriction enzyme used. Faint traces of such subfragments were occasionally present in DNA from untreated cells (for example, Fig. 1*d*), consistent with the presence of a small subpopulation of endogenously activated cells in the untreated population^{7,9}.

The occurrence of such a subfragment after mild nucleolytic digestion of chromatin indicates that a specific site in the vicinity

of the C_κ loci in treated cells is unusually sensitive to DNase action, being cleaved by the enzyme with extremely high frequency. We have mapped the position of this hypersensitive site relative to several neighbouring restriction enzyme sites, and have identified its location in the known restriction map

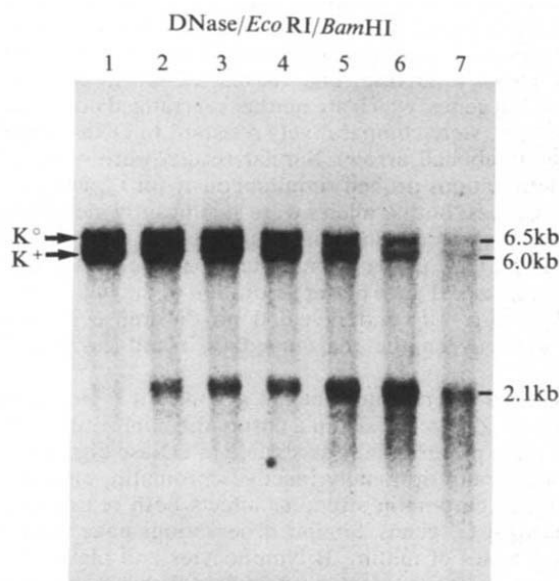


Fig. 2 Nuclease digestion of κ genes from cells treated with LPS. Growth conditions were the same as described in Fig. 1 legend, except that LPS from *Salmonella typhosa* 0901 (Difco) was included ($10 \mu\text{g ml}^{-1}$) for 20 h before collection. These conditions gave maximal expression of surface κ determinants and of κ -specific RNA, but did not affect the doubling time of the cells (12 h). DNA was isolated from nuclei of these cells after treatment with DNase (see Fig. 1 legend). The extent of bulk DNA digestion varied rather between nuclear preparations; the samples shown here were digested to approximately half the extent of corresponding lanes in Fig. 1. After combined *EcoRI*–*BamHI*, the purified DNA was electrophoresed, transferred to nitrocellulose, and probed for κ constant region sequences. K° and K^+ denote the embryonic and rearranged κ loci, respectively.

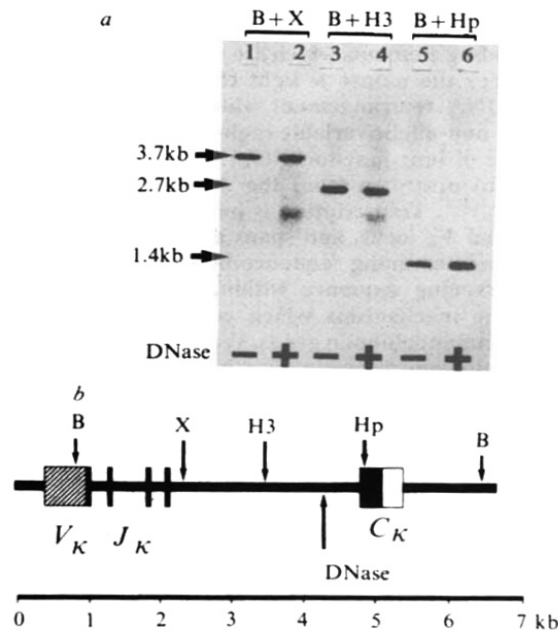


Fig. 3 Location of the LPS-inducible hypersensitive site. DNA was isolated from the nuclei of LPS-treated cells either with or without previous DNase digestion. The DNA was cleaved first with *BamHI*, then with one of several other restriction enzymes, electrophoresed, transferred to nitrocellulose, and probed for κ constant region sequences. *a*, The location of the hypersensitive site was mapped by comparing the sizes of fragments generated with and without DNase for each pair of restriction cleavages. The 2.1-kb subfragment appears in lanes 2 and 4. No differences in the restriction digestion patterns of the two alleles were noted for regions downstream of the J_κ loci. *b*, The map shows the rearranged κ allele; the structure of this gene was determined by analysis of recombinant phage carrying the rearranged κ locus, cloned from 70Z/3 cells³². C_κ , constant coding and 3' untranslated regions; J_κ , joining region coding elements; V_κ , 5' untranslated and variable region coding sequences; B, *BamHI*; H3, *HindIII*; Hp, *HpaI*; X, *XbaI*; DNase, the inducible hypersensitive site.

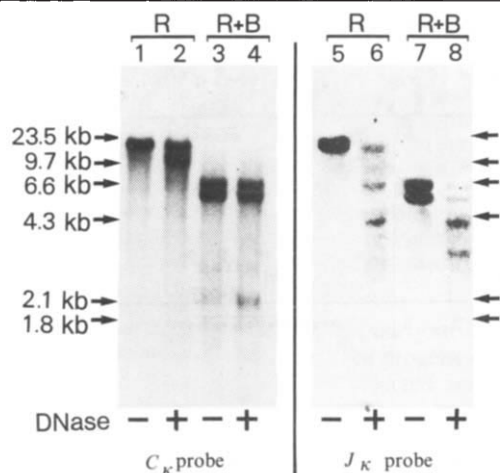


Fig. 4 Boundaries of the inducible hypersensitive site. DNA was isolated from LPS-treated cells either with or without previous DNase digestion of nuclei. After cleavage with either *Eco*RI alone (R) or a combination of *Eco*RI and *Bam*HI (R+B), the DNA was electrophoresed, transferred to nitrocellulose, and probed for either κ constant region (lanes 1–4) or J_κ (lanes 5–8) sequences. The J_κ probe was a 2.5-kb *Hind*III fragment of the embryonic κ locus¹⁵, isolated from a recombinant plasmid provided by E. Max and P. Leder. DNase concentrations were 1.0 U ml^{-1} in lanes 2 and 4, and 3.0 U ml^{-1} in lanes 6 and 8. The approximate sizes of the subfragments generated by DNase digestion are: lane 2, 10 kb; lane 4, 2.1 kb; lane 6, 6.5 and 4.2 kb; lane 8, 4.2 and 3.7 kb.

of the C_κ region¹⁵ (Fig. 3). The hypersensitive site lies inside the region which forms the large intervening sequence of a rearranged κ gene, with its 3' boundary 0.5–0.7 kb upstream of the constant region coding sequence. To identify the 5' boundary of the hypersensitive site, restriction digests of DNA from LPS-treated cells were probed for fragments bearing the J_κ region (Fig. 4). Mild DNase digestion always produced two distinct J_κ -bearing subfragments, corresponding to the two κ alleles. Analysis of the sizes of these subfragments indicated that DNase hypersensitivity arises at the same location in both the rearranged and germline κ alleles after exposure to LPS, and is confined to a discrete region not more than 0.5 kb long. The location of this hypersensitivity does not coincide with known sites of transcriptional initiation, DNA recombination^{16,17} or RNA splicing, and serves no other recognized function. Recently, however, the sequence of a short (150-base pair) segment of DNA occupying approximately this same location, has been found to be conserved evolutionarily between the human and mouse κ genes, whereas the remaining intronic sequences on either side have undergone extensive divergence¹⁸. This segment is, in fact, the only conserved noncoding region in the vicinity of the C_κ locus. No additional hypersensitive sites were detected; in particular, there was no evidence of hypersensitivity near the 5' end of the rearranged κ gene. We have detected neither constitutive nor inducible transcripts derived from the germ-line κ locus in these cells, suggesting that hypersensitivity is not simply a consequence of ongoing transcription (data not shown). The factors which prevent coordinate activation of the germ-line allele remain to be elucidated.

Thus, our data indicate that when κ light chain expression is induced by LPS treatment, a discrete DNA region closely linked to the constant region coding sequence becomes extremely sensitive to DNase digestion, presumably as a result of a localized change in chromatin structure. While this region has no identified function, its sequence has apparently been conserved during evolution, whereas adjacent sequences have not. Similar hypersensitive sites have been observed in other genes^{19–25}, where they are frequently (but not exclusively) found near the transcriptional initiation site at the 5' end of the gene. Because the hypersensitivity of certain of the sites correlates with gene activity, it has been argued that these may have a role in the regulation of transcription^{20–25}. By analogy with these sites, it is tempting to speculate that the LPS-inducible hypersensitive site found inside the κ gene may play a part in

transcriptional activation of the gene in response to lipopolysaccharide.

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Genomic environment of the expression-linked extra copies of genes for surface antigens of *Trypanosoma brucei* resembles the end of a chromosome

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The surface coat of trypanosomes consists essentially of a single protein, the variant surface glycoprotein (VSG)^{1,2}. But more than a hundred different VSGs can be produced³. By switching from the synthesis of one VSG to the next, trypanosomes change the antigenic nature of their surface and thus escape destruction by the host immune system^{4–6}. Each VSG is encoded by a separate gene and expression of some of these genes involves the duplication and transposition of a silent basic copy (BC) into a new genomic environment^{7–9}, where the gene is transcribed^{10,11}. The DNA segment downstream of this transposed expression-linked extra copy (ELC) of the gene has two remarkable properties. It is devoid of restriction endonuclease cutting sites for a stretch of 7 kilobases (kb) which ends abruptly in an apparent cluster of at least 17 restriction endonuclease cutting sites. Here we show that in intact DNA the 'barren' region 3' to two active VSG genes is preferentially attacked by exonuclease *Bal*31. We conclude that the expressed copies of these genes are located adjacent to a discontinuity in the DNA, presumably the end of a chromosome.

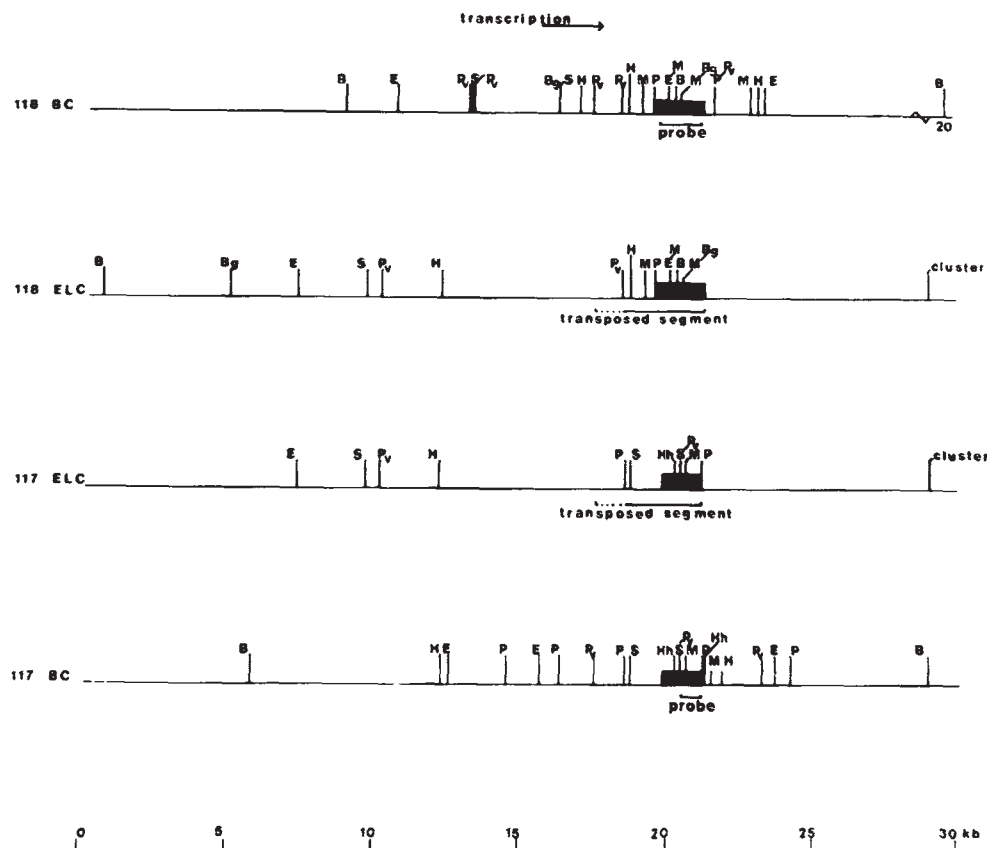


Fig. 1 Physical maps of the 117 and 118 VSG genes. Restriction endonuclease cleavage sites in and around the BC and ELC genes for VSGs 117 and 118 in *Trypanosoma brucei* (from ref. 12). The clusters at the 3' ends of the ELC maps indicate small regions where 17 restriction enzymes appear to cut (*EcoRI*, *BamHI*, *PstI*, *MspI*, *HindIII*, *PvuII*, *BglII*, *SalI*, *HhaI*, *HphI*, *TaqI*, *XbaI*, *BspI*, *XhoI*, *AvaI*, *KpnI*, *MboI*). The transposed segment of the BC genes is shown in the ELC maps. The sequences that hybridize to the probes used in the experiments shown in Fig. 2 are indicated underneath the BC genes. Abbreviations: B, *BamHI*; Bg, *BglII*; E, *EcoRI*; Hh, *HhaI*; H, *HindIII*; M, *MspI*; P, *PstI*; Pv, *PvuII*; S, *SalI*.

The physical maps of the BCs and ELCs of VSG genes 117 and 118 are shown in Fig. 1. The regions surrounding both ELCs are indistinguishable and both genes may have been inserted into the same expression site. This site is characterized by long stretches of DNA in which no restriction enzymes cut. These stretches may represent repetitive DNA, but this has not been verified, as all attempts to isolate this DNA as recombinant DNA have failed^{11,12}.

To test whether the apparent restriction site cluster downstream of the ELCs really is a discontinuity in the DNA, we determined whether this site is preferentially attacked by *Bal31* exonuclease in large DNA. DNA with a molecular weight of 50–80 kb was isolated from trypanosome variant antigen types 117 and 118 and incubated with *Bal31* for various periods of time. The DNA was recovered and digested with appropriate restriction endonucleases to examine the effect of *Bal31* digestion on ELC and BC fragments in Southern blots.

Figure 2a shows a Southern blot of 117 DNA treated with *Bal31* and restricted with *PvuII*. The filter-bound DNA was hybridized to the 3' part of a cloned VSG 117 complementary DNA (cDNA) fragment. Two restriction fragments are detected: a 2.7-kb BC gene fragment and a 8.7-kb fragment that represents the ELC gene. The ELC gene fragment is progressively shortened by *Bal31*, whereas the BC gene fragment is unaffected even by the most extensive *Bal31* treatment.

The probe used to visualize the 117 VSG gene fragments is known to cross-hybridize with a family of putative VSG genes, the 117-related genes^{6–8,12}. Even at the high stringency used in this experiment ($0.1 \times \text{SSC}$ at 65°C), 117-related genes are detected. All cross-hybridizing fragments, some of which are larger than the 117 ELC gene fragment, are resistant to *Bal31* digestion, suggesting that the shortening of the 117 ELC gene fragment by *Bal31* nuclease is a specific event, unrelated to the size of the target.

Figure 2b shows the result of an analogous experiment with variant type 118 DNA. After *Bal31* treatment, the DNA samples were cleaved with *BglII* and a Southern blot of this

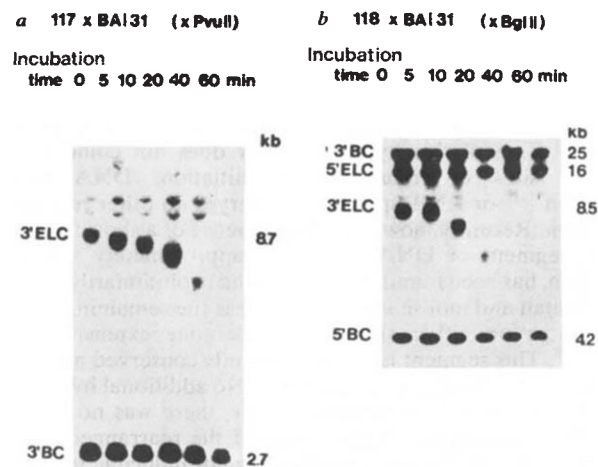


Fig. 2 Autoradiograms showing the preferential sensitivity of the 3' flanking region of the 117 and 118 ELC genes to digestion by *Bal31* nuclease. The DNA from trypanosome variant types 117 and 118 (MITat 1.4 and 1.5) was isolated as described in ref. 11 and digested with 1 U *Bal31* nuclease (Biolabs) per $30 \mu\text{g}$ DNA, as described in ref. 13, for the times indicated. The DNA was collected by ethanol precipitation after phenol extraction and digested with the restriction enzymes indicated. The restricted DNA was size-fractionated on agarose slab gels, blotted onto nitrocellulose filters and hybridized to nick-translated ^{32}P -labelled VSG cDNA fragments. *a*, Southern blot of 117 DNA treated with *Bal31*, digested with *PvuII* and hybridized to the 3' *SalI*-*PstI* fragment from TcV117-5 (ref. 11). *b*, Southern blot of 118 DNA treated with *Bal31*, digested with *BglII* and hybridized to the *PstI* insert fragment of TcV118-2 (ref. 11). The localization of the genomic sequences that hybridize to the cDNA fragments is shown in Fig. 1. The origin and size of the fragments detected are indicated. The extra hybridizing fragments in *a* represent 117-related genes (see text).

DNA was probed with a cloned VSG 118 cDNA fragment. One of the four *Bgl*II fragments detected is shortened by *Bal*31 nuclease. This 8.5-kb *Bgl*II fragment contains the 3' part of the 118 ELC gene and its flanking region.

The preferential sensitivity to *Bal*31 digestion of the 3'-flanking region of the 117 and 118 ELC genes shows that this region contains a discontinuity. As the *Bal*31 preparations also cleave single-stranded DNA¹⁴, this discontinuity could either be in one DNA strand (nick or gap) or in both DNA strands. The fact that 17 restriction endonucleases appear to cut at this position is compatible only with a discontinuity in both strands. Whether this discontinuity is also present *in vivo* or unmasked by removal of protein or RNA during DNA isolation is not easily tested with trypanosomes, because they lack condensed or polytene chromosomes suitable for cytological (hybridization) analysis.

The ends of the linear DNA molecules found in viruses or chromosomes are known to have unusual properties. A protein may be covalently linked to the end^{15,16} or the two DNA strands may be linked and form a hairpin¹⁷⁻²¹. The presence of a long hairpin (more than 500 base pairs) in the trypanosome DNA was rendered unlikely by running restriction digests of nuclear DNA on an alkaline gel before and after treatment with *S*₁ nuclease, which specifically cuts single-stranded DNA. No shortening of ELC fragments was seen (experiment not shown).

Another unusual feature of chromosome ends is the presence of tandem repeats. These were first detected at the ends of ribosomal DNA molecules, which were shown to contain 20-70 tandem repeats of the hexanucleotide CCCC¹⁶. This simple repeated sequence is probably present at most termini of *Tetrahymena* macronuclear DNA²² and analogous short terminal repeats have been found in other ciliates²³⁻²⁵, in the rDNA of the slime mould *Dictyostelium*²⁶ and in linear viral DNAs^{21,27}. The recent finding that the ends of rDNA from *Tetrahymena* can be stably replicated in yeast nuclei as ends of linear plasmids²⁸ suggests that terminal repeats may indeed be a common characteristic of most stable linear nuclear DNAs. The 'barren' regions surrounding the ELCs (Fig. 1) could, therefore, represent analogous short tandem repeats marking the end of a chromosome.

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A specific replication origin in the chromosomal rDNA of *Lytechinus variegatus*

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Specific replication origins have been well characterized in prokaryotes^{1,2}, and have been identified in eukaryotic extra-chromosomal DNAs³⁻⁸. The best candidate, until now, for a specific eukaryotic chromosomal replication origin has been found in yeast⁹, but the general existence of such origins is challenged¹⁰ by the finding that SV40 and polyoma DNA fragments lacking the viral replication origin can replicate after injection into *Xenopus* eggs. It has thus been suggested that the origins found on extrachromosomal DNAs exist solely to circumvent the cells' strict requirement for chromosomal DNA to replicate once per cell division. Clearly the disparate views can only be reconciled after the replication of specific chromosomal genes has been studied. To this end, we have studied the replication of the ribosomal genes (rDNA) of the sea urchin *Lytechinus variegatus*. The visualization of replicating rDNA, isolated from rapidly dividing sea urchin gastrula cells, after restriction endonuclease digestion demonstrates that the initiation of replication in these chromosomal genes is sequence-specific, and is most probably confined to a region within the non-transcribed spacer.

After fertilization *L. variegatus* undergoes a rapid cleavage stage during which the embryos divide approximately every half hour. The rDNA genes of *L. variegatus* are present in about 200 copies per genome, and are isolatable as a dense satellite after equilibrium density centrifugation in CsCl^{11,12}. DNA was isolated from sea urchin embryos about 4 h after fertilization. Following the first CsCl equilibrium density gradient, the ribosomal DNA peak was located by hybridization with a cloned ribosomal gene (pUNC220, provided by Dr D. Stafford). The rDNA was then recentrifuged in a second CsCl gradient and found to band at a density of 1.723 g cm⁻³. The rDNA peak formed a dense satellite well separated from the main band DNA, which has a mean density of about 1.695 g cm⁻³ (ref. 11).

Table 1 Purity of rDNA fractions

Hours	% Chromosomal DNA bound at equilibrium	
	Expt 1	Expt 2
24	17.5	18.4
48	15.7	14.6
72	14.0	14.3

The purity of ribosomal fraction was determined by saturation hybridization: three plasmids with inserts spanning the ribosomal repeat (see Fig. 1) were pooled on an equal weight basis, simultaneously nicked and denatured by treatment with 0.2 M NaOH for 10 min at 65 °C, neutralized and 10 µg applied to each 2.5-cm filter. Filters were cut into four identical pieces and after 1 h at 75 °C in 0.2 × SSC, 0.1% SDS, each was added to 56% (v/v) formamide, 4.6 × SSC, 9.2% dextran sulphate, 0.1% SDS containing about 2 ng ³²P-labelled chromosomal DNA and 0.5 ng ³H-labelled nick-translated 'pooled plasmid' as an internal control. Hybridization was at 42 °C for the indicated times. Binding of the chromosomal probe and the pooled plasmid was found to be invariant over a 40- and 14-fold range, respectively. Results are normalized to the fraction of the ³H-labelled pooled plasmid internal control at each time point. In both experiments, the value at 4 h was 20% but this time is omitted because it is pre-equilibrium. Maximum hybridization of the control was 57% of input for expt 1, 58% of input for expt 2.

The purity of the rDNA fraction was assayed by electron microscopy (EM) and saturation hybridization. When hybridized to a vast excess of cloned rDNA fixed to nitrocellulose, the chromosomal rDNA fraction was judged to be about 18% pure (Table 1). The EM determination is based on the measurement of DNA size after endonuclease restriction with an enzyme which recognizes one site in each rDNA repeat¹¹. Based on the fraction of molecules in the rDNA size range (see below and ref. 1), we estimate that the rDNA fraction was 23% pure.

The rRNA genes have been shown to occur as tandem repeats separated by an untranscribed spacer region in a variety of eukaryotes^{11,13-16}. Since the rDNA gene region usually covers more than 10⁶ base pairs (bp) there is a very great likelihood that some of the gene repeats will contain origins of replication. An additional feature of the gene family is that the repeat unit can vary in size within any one species¹⁷. This is due to the fact that the spacer region between transcription units varies in size. In *L. variegatus*, a spacer size variation of about 2 kilobase pairs (kb) has been reported (D. Stafford, personal communication, and ref. 11) as shown in Fig. 1. We have estimated the range of repeat sizes by restricting rDNA with the single cutter *Kpn*I, sizing the digest by agarose gel electrophoresis and blotting the gel to nitrocellulose. Hybridization of the blot with cloned rDNA shows most of the variation in size to be between 11 and 14 kb (Fig. 2). However, approximately 13% of the DNA has a size of about 9 kb.

To determine the position of the replication origins, the rDNA was restricted with *Kpn*I and visualized by EM. Before restriction, about 2% of the molecules contained eye forms¹⁸. After restriction, less than 1% contained eye forms (Fig. 3). A total of 128 molecules with eye forms were found; of these, 25 were in the ribosomal size range between 10.8 and 13.8 kb. In Fig. 4, the position of the replication bubbles on these molecules is indicated. Each molecule has one end placed at 0 kb, and has been oriented so that the centre of the replication loop (the origin) is to the right of the centre of the molecule.

It is not possible to discern the orientation of a given molecule visualized in the electron microscope by our techniques. However, it is also not possible to orient a series of such molecules to give a tight clustering of replication origins (assuming that the molecules are displayed with one end aligned) if the origins are randomly distributed along the molecules. Thus, if the members of a sufficiently large sample can be oriented to produce a tight distribution of origins, one can conclude that the origins are indeed located specifically with respect to sequence. A broad distribution, on the other hand, would most probably indicate a location random with respect to sequence.

Plots of the distances from the *Kpn*I site to the midpoint of the replication eyes are shown in Fig. 5. When the midpoints are plotted using the long distance to the *Kpn*I site (Fig. 5a), a tight distribution is found. Thus the origins are located specifically with respect to sequence.

The centre of the distribution is 8.4–8.6 kb from the *Kpn*I site. This places it either within the 26S gene, or within the non-transcribed spacer 2 kb from the 3' end of the 26S gene. Should the midpoint occur within the 26S gene, the short distance to the *Kpn*I site would be constant from molecule to molecule and the long distance would vary. Conversely, should the midpoint occur within the spacer, the long distance would

be relatively constant from molecule to molecule and the short distance would vary. In Fig. 5b, the short distance from the midpoint of the eyes to the *Kpn*I site is charted. A broad, random distribution is clearly seen in this case. Thus we tentatively assign the replication origin to the non-transcribed spacer, about 2 kb from the 3' end of the 26S gene (Fig. 1).

We note that several assumptions have been made in our analysis of the data. First, we assume that chromosomal replication is bidirectional. The available evidence indicates that this is a valid assumption¹⁹⁻²¹. Second, we have treated all molecules between 10.8 and 13.8 kb as though they were of ribosomal origin. While undoubtedly some of the molecules are contaminants, it is likely that the large majority are ribosomal, as indicated by the agreement between the saturation hybridiz-

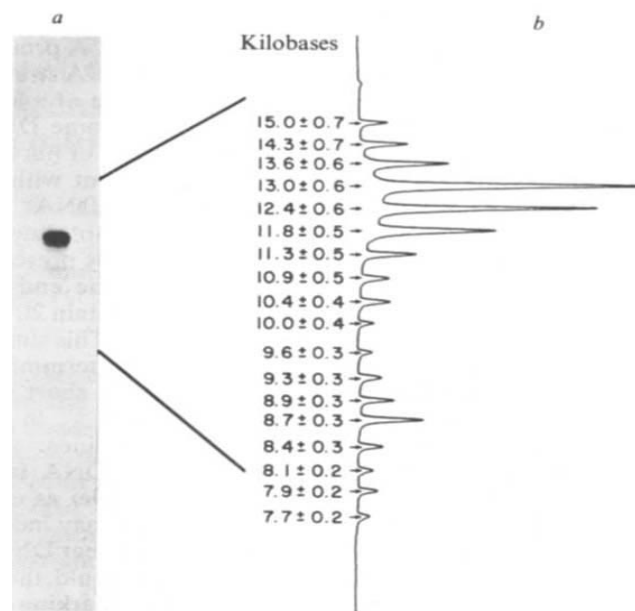


Fig. 2 Gulf Coast *L. variegatus* were obtained from the Gulf Specimen Company, Panacea, Florida, during season (May–June). 6 ml of packed embryos were chilled at the eighth cleavage stage (~4 h after *in vivo* fertilization) and allowed to settle for 25 min at 1 g on ice. After washing in 250 ml ice-cold seawater and resettling, they were added to 50 ml of cold buffer containing 33 g CsCl, 10 mM 2-mercaptoethanol, 20 mM Tris-HCl, pH 8.0, 50 mM EDTA and immediately ruptured by several passes of a dounce homogenizer. The homogenate was adjusted to a density of 1.499 g cm⁻³ and centrifuged for 90 min at 20,000 r.p.m., 20 °C in a SW27 rotor. The protein layer was removed and the remainder was adjusted to a density of 1.6 g cm⁻³, layered on a cushion of CsCl and centrifuged as above for 14 h. Fractions containing DNA were pooled and banded by CsCl equilibrium density centrifugation for 3 days at 20 °C, 35,000 r.p.m. in a Ti60 rotor. Aliquots of each fraction were denatured and bound to nitrocellulose and rDNA detected by hybridization with a nick-translated rDNA clone, pUNC220. The rDNA was then rebanded in a second CsCl gradient. The ribosomal peak was then dialysed overnight against 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM NaCl. Approximately 0.1 µg of purified rDNA was digested with *Kpn*I at 30 °C in the presence of 0.3 µg λ *Hind*III fragments to assess the completeness of the digestion conditions. Samples were electrophoresed in a 0.6% agarose gel containing ethidium bromide. The gel was blotted by Southern's procedure³¹ and hybridized with a nick-translated set of pooled plasmids containing inserts spanning the entire *Lytechinus* ribosomal repeat (pUNC220, pLv147 and pLv128, courtesy of Dr Stafford). The filter was washed in 0.1 M NaCl, 0.1 mM EDTA, 0.1% SDS at 68 °C, cut into consecutive 1-mm slices which were individually autoradiographed and then scanned by densitometry to give a quantitative measure of the length of distribution of the *Kpn*I-digested rDNA unbiased by the spread of ³²P radiation. *a*, Autoradiogram of the intact blot; *b*, densitometer tracings of consecutive 1-mm slices of the blot.

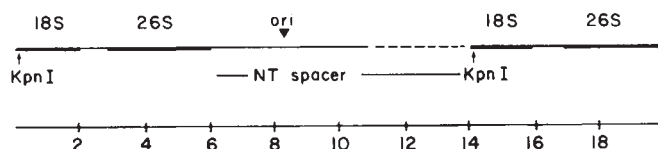
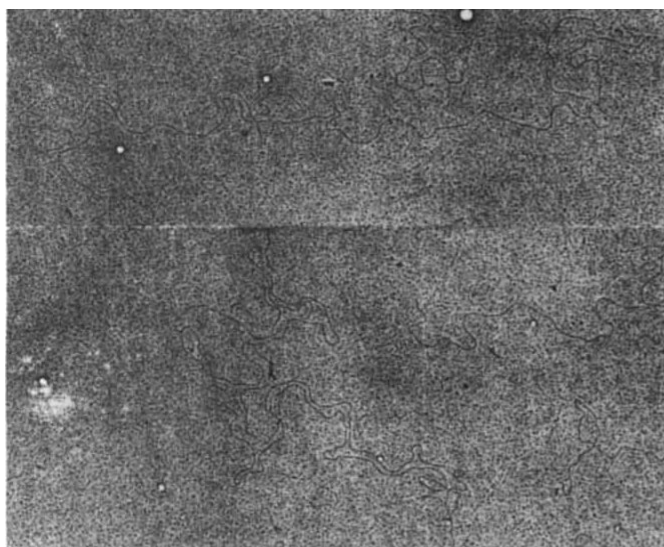
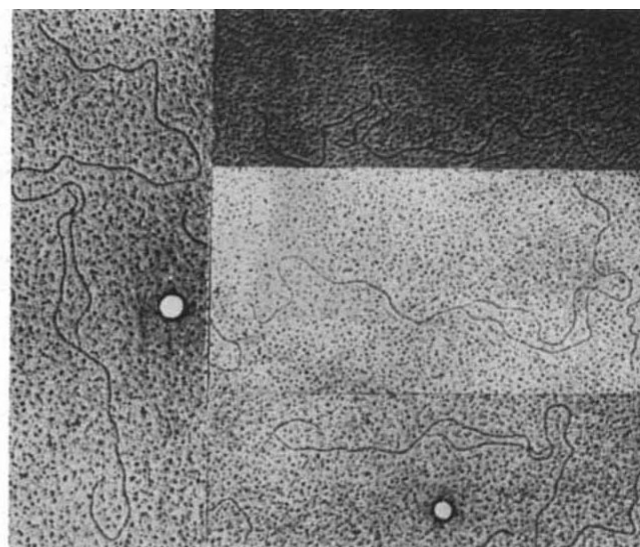


Fig. 1 The rDNA repeat in *L. variegatus*. The dashed line in the non-transcribed spacer region indicates the approximate amount of heterogeneity among the various members of the class, not the location of the heterogeneity, which is spread throughout the non-transcribed spacer region.

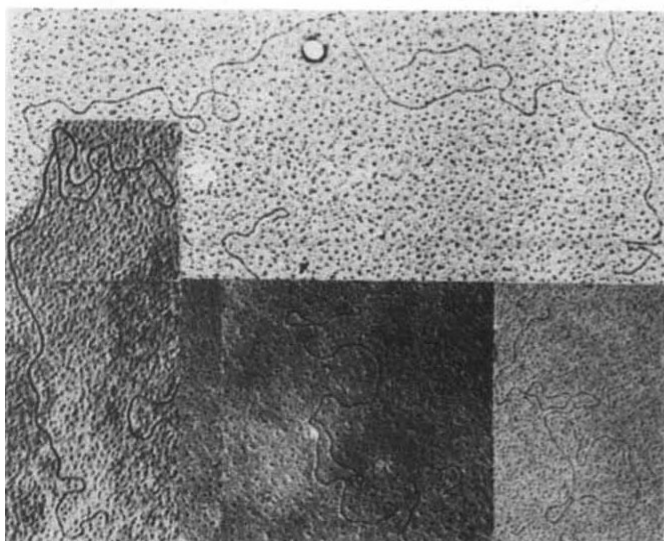


a



c

Fig. 3 Eye forms before and after *Kpn*I digestion. DNA was spread essentially as described by Davis³². The spreading solution and the hypophase contained, respectively, 40% and 10% formamide. DNA was digested with *Kpn*I as described in Fig. 1 legend and diluted 20–50-fold into spreading solution. pBR322 was added as a molecular weight standard. *a*, Uncut DNA; *b*, eye form in the rDNA size range; *c*, eye form in the 9–10.3 kb size range.



b

ation, 18% and the per cent of total eyes in the right size range, 23%.

It remains to be determined whether every ribosomal gene contains an origin. After fertilization, *L. variegatus* undergoes a rapid cleavage stage during which the embryos divide every half hour. At this stage the entire sea urchin genome is dividing at the rate of *Escherichia coli*, even though it contains several orders of magnitude more DNA. Based on a replication rate of about 3 kb per min per fork, *L. variegatus* should contain about 10,000 forks, or one eye form every 10^5 bp¹³. This translates into about one eye form per 10 ribosomal genes. Theoretically it is possible that only one out of 10 spacers contains an origin or that most spacers contain origins and the ones used are chosen at random.

A second class of molecules with a size distribution of 9 to 10.3 kb has also been found to contain a specific origin of replication (Fig. 4). The replication origin of these molecules maps at about 6.1 kb from one *Kpn*I site (Fig. 5c). About 12% of the total eye forms visualized were on 9–10.3-kb sized molecules. While we have observed rDNA in this size range, it is unlikely that the amount observed can account for 10% of the total eyes seen or almost 50% of the major class of rDNA genes. A possible alternative is that these molecules represent a separate chromosomal region with a defined origin of replication about 6.1 kb from a *Kpn*I site. We note, however, that if this class were ribosomal, the origin would occur exactly

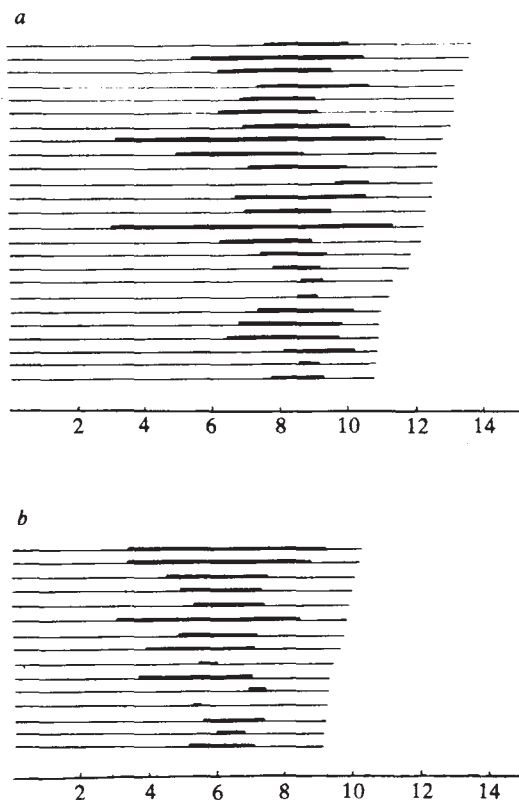


Fig. 4 Position of the eye forms on: *a*, molecules between 10.8 and 13.8 kb; *b*, 9 and 10.3 kb.

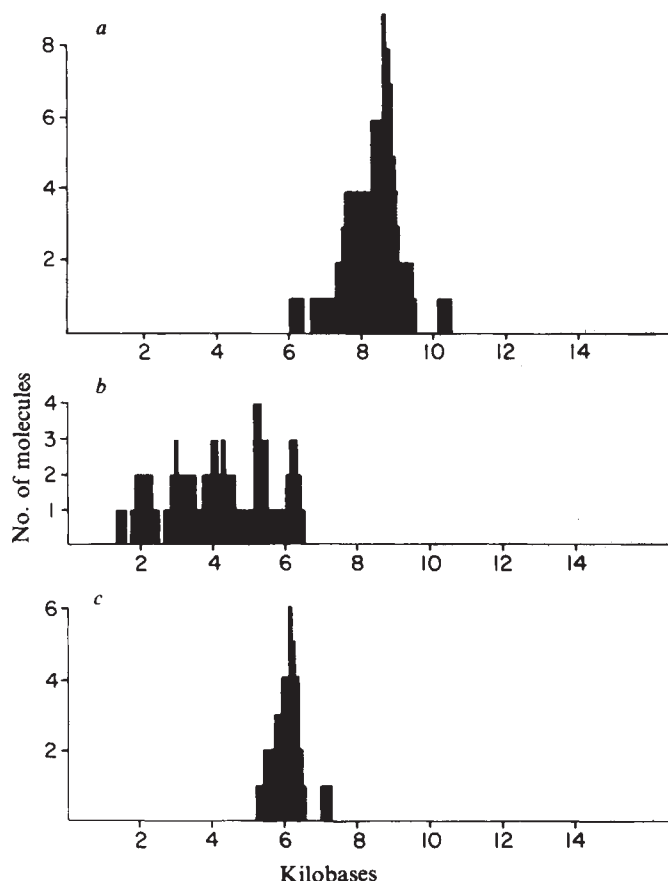


Fig. 5 Mapping of the replication start sites. The distance from the midpoint of the eye forms to the ends of each molecule was measured. A 3% error correction, determined by successive measurements of the standard, pBR322, was incorporated into each determination. *a*, The longer distance from the midpoint of the eyes to the *KpnI* site on molecules between 10.8 and 13.8 kb; *b*, the shorter distance from the midpoint to the *KpnI* site; *c*, the eye midpoint on molecules between 9 and 10.3 kb.

at the boundary between the 26S gene and the non-transcribed spacer, a distance that is 6.15 kb from the *KpnI* site (Fig. 1).

The finding of a specific replication origin for the rDNA genes of *L. variegatus* is consistent with previous work in prokaryotes and the localization of origins in lower eukaryotic extrachromosomal elements. In addition to the finding of specificity, the suggested localization within the rDNA spacer is consistent with the assignment of the other mapped origins to non-transcribed regions. The amplified rRNA genes in *Tetrahymena*^{6,22} and *Physarum*⁷ have specific origins of replication mapping near the middle of the spacer separating the linear, palindromic genes. Using Miller spreads of ribonuclear complexes, the ribosomal genes of *Drosophila* showed a negative correlation between the centre of eye forms and transcription fibrils, suggesting that the origin resides in the spacer region²³. In yeast, autonomously replicating plasmids (ARS) with specific chromosomal inserts have been characterized by a higher transformation frequency than uninserted vectors, a closed circular form, and a high rate of curing^{9,24}. The putative origin of the yeast ARS element containing the *TrpI* gene maps in a non-transcribed region flanking the 3' end of the gene^{25,26}. The origin in *Xenopus* mitochondrial DNA also maps to a region in which no transcripts have been detected²⁷. If origins in general are located in spacer regions, then the compelling need for an RNA primer to accompany replication must be resolved with the absence of spacer transcription. The promoters located in some spacers could serve a replication function²⁸⁻³⁰.

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Suppression of an amber mutation by microinjection of suppressor tRNA in *C. elegans*

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Informational suppression by nonsense suppressor tRNAs has classically been a powerful tool for study of the mechanism of protein synthesis, to obtain conditional mutants and to demonstrate that a gene encodes a given protein product (for reviews see refs 1-3). In the nematode *Caenorhabditis elegans*, two genetically identified suppressors, *sup-5* and *sup-7* (refs 4, 5), have recently been shown to be amber suppressor tRNAs (N. Wills, R. F. Gesteland, J. Karn, L. Barnett, S. Bolten and R. H. Waterston, personal communication). We report here the microinjection of *sup-7* tRNA into the gonad of an animal bearing an amber allele of a maternal-effect mutant affecting sex determination (*tra-3*). We observe phenotypic suppression in the injected parent's offspring. tRNA from wild-type animals does not show this *in vivo* suppressor activity, and *sup-7* tRNA does not cause suppression of a non-amber allele of the same gene. *In vivo* suppression of an amber mutant by microinjection provides a new means of gene manipulation in *C. elegans*.

tRNA was introduced into *C. elegans* embryos by injection into the hermaphrodite gonad (Fig. 1). In this technique, a micropipette penetrates the adult cuticle and body wall to enter the distal arm of the gonadal tube (~400 µm long and 30 µm in diameter, Fig. 1a). This region of the gonad consists primarily of immature germ cells connected by bridges to a central core of cytoplasm⁶ (Fig. 1b). Studies with a fluorescein-tagged D-amino acid dodecapeptide (synthesized and kindly provided by D. Weisblatt) demonstrate that small injected macromolecules

Fig. 1 Introduction of tRNA into embryos by microinjection into the parental gonad. The hermaphrodite gonad consists of two symmetrical and equivalent U-shaped tubes⁶. Each half produces oocytes and sperm; embryos are made in assembly-line fashion as oocytes pass, one by one into the spermatheca to be fertilized, and then enter the uterus to begin embryogenesis. A micropipette (4–6 μm diameter at the tip) is inserted into the germ-line tissue in the distal arm of the gonad of an animal anaesthetized with 0.01% tetramisole and 0.1% tricaine mounted against the edge of a coverslip under Voltalef oil (BDH), and inspected using a Wild M8 dissecting microscope at $\times 50$ magnification. 5–10 pl tRNA (20 mg ml⁻¹) is injected using an oil-filled syringe connected to the micropipette. Each injected worm is examined after 4–8 h to ensure that the injected half-gonad is still producing embryos. Embryos made by the uninjected half-gonad serve as an internal control.

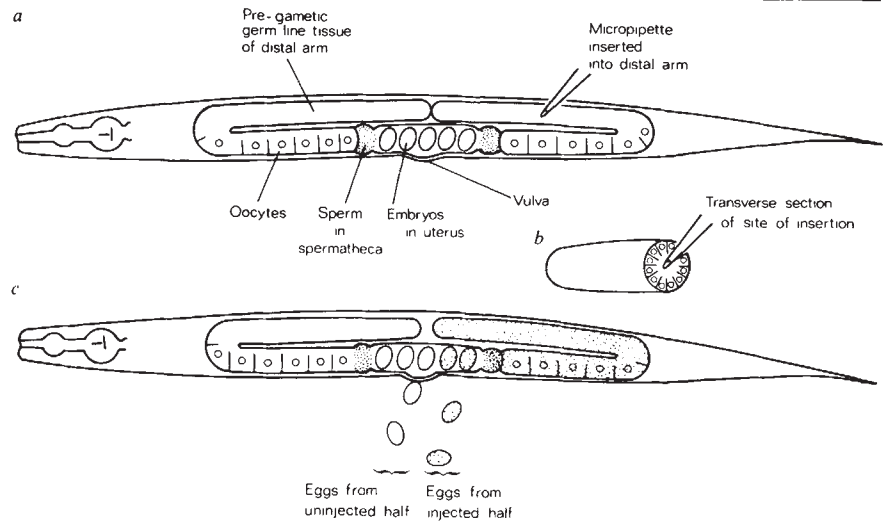
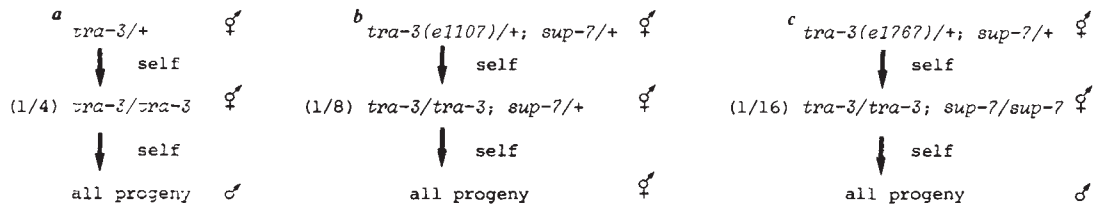


Fig. 2 a, Maternal effect of *tra-3*⁷. Homozygous *tra-3/tra-3* XX animals are hermaphrodite (♀) if born from a heterozygous *tra-3/+* parent, but are pseudomale (♂) if born from a homozygous *tra-3/tra-3* parent.



In addition, a wild-type gene introduced by cross-fertilization by a wild-type male produces hermaphrodite cross-progeny, again rescuing the *tra* phenotype. **b**, One allele of *tra-3*, *e1107*, is suppressed. Here, we show that one maternal dose of *sup-7* is sufficient for suppression. Other crosses have shown that one copy of *sup-5* or *sup-7* in either the hermaphrodite parent or the zygote can rescue the *e1107* phenotype. **c**, Another allele of *tra-3*, *e1767*, is not suppressed. Two copies of *sup-7* in both parent and zygote cannot rescue the *e1767* phenotype.

can diffuse within minutes throughout the U-shaped gonadal tube and that oocytes and embryos produced by this tube contain injected tracer (data not shown). Thus, this procedure provides a means of injecting several embryos by a single injection into a target tissue that is large and easy to inject compared with the embryo (Fig. 1c).

The mutation selected for attempted rescue by microinjection of *sup-7* tRNA is in the *tra-3* locus⁷. Normally in *C. elegans*, sex is determined by the X:autosome ratio¹¹. In a wild-type diploid strain, XX animals are self-fertilizing hermaphrodites and XO animals are male. However, recessive mutations in one of three autosomal transformer genes (*tra-1*, *tra-2* and *tra-3*) divert XX animals from the hermaphrodite to the male pathway of development⁷.

tra-3 was chosen for mutant rescue by injection of suppressor tRNA for two reasons. First *tra-3* exhibits a maternal effect (Fig. 2a). The wild-type gene product expressed by a heterozygous (*tra-3/+*) XX hermaphrodite rescues its homozygous (*tra-3/tra-3*) self progeny. The self progeny of these *tra-3/tra-3* hermaphrodites, however, all develop as morphologically distinct pseudomales (Fig. 3a). The first sign of sexual dimorphism is midway during embryogenesis (J. Sulston, personal communication). Thus, the *tra-3* gene product is likely to be present in oocytes, and must function during embryogenesis.

Second, both amber and non-amber alleles of *tra-3* are available. Genetic tests with *sup-5* and *sup-7*, both amber suppressors, have shown that *e1107* is an amber allele of *tra-3* and that *e1767* is not (Fig. 2b,c). (Alleles are given lettered numbers, for example, *e1107*, according to the place and time isolated⁸.) Even a single copy of the *sup-5* gene, the weaker of the two suppressors⁵, is sufficient to rescue the *tra-3* phenotype of *e1107* progeny (Fig. 2b). Conversely, two copies of the stronger suppressor, *sup-7*, do not rescue the *tra-3* phenotype of *e1767* progeny (Fig. 2c).

sup-7 tRNA (for extraction procedure see Table 1 legend) was microinjected into the distal arm of *e1107/e1107* hermaphrodite gonads, and the progeny of each injected parent examined for suppression. The tail, vulva and gonad morphology of a hermaphrodite is easily distinguishable from that of the expected *tra-3* pseudomale. Ten animals continued to produce embryos in the half-gonad injected. Of these, six produced some progeny that were clearly hermaphrodite (Table 1). The presence of any hermaphrodites among progeny of a

Table 1 Results of suppressor tRNA microinjection in *C. elegans*

Allele injected	tRNA injected	No. injected*	No. progeny†	No. hermaphrodites
<i>e1107</i>	<i>sup-7</i>	10	1,023	21
<i>e1767</i>	<i>sup-7</i>	10	1,106	0
<i>e1107</i>	Wild type	13	1,127	0
<i>e1107</i>	—	—	4,000	0

Wild-type (*C. elegans* Bristol strain, N2) and *sup-7*-bearing [RW2108 = *unc-15(e1214)*I; *dpy-18(e364)*III; *sup-7(st5)*X] animals grown in liquid culture⁹ were suspended in 0.05 M disodium naphthalene disulphonate together with an equal volume of phenol saturated with 1% aqueous 8-hydroxyquinoline and disrupted in a French pressure cell at 2,000 p.s.i. RNA was extracted according to the method of Kirby¹⁰ and fractionated on a DEAE-cellulose (Whatman DE52) column. The fraction eluting between 0.25 M and 1.0 M NaCl, containing 4S, 5S and small RNAs, was assayed for tRNAs by aminoacylation with ¹⁴C-labelled amino acids, using a crude nematode aminoacyl tRNA synthetase preparation. Using a mixture of 10 ¹⁴C-labelled amino acids (each 100 cmol⁻¹), 300–500 pmol per A₂₆₀ unit of both wild-type and *sup-7* crude tRNAs were charged.

* Only animals that continue to produce embryos in the half-gonad injected are included.

† Only half the number listed arise from the injected half-gonad.

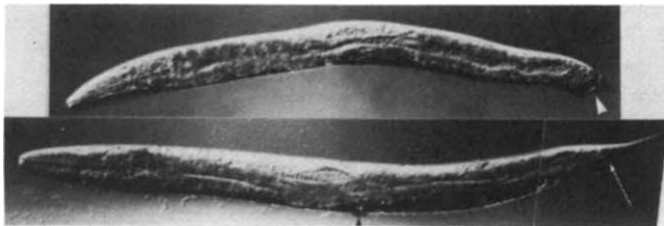


Fig. 3 Comparison of the unrescued *tra-3* phenotype, a pseudomale (a), and a rescued hermaphrodite (b). Both animals were derived from a *tra-3/tra-3* XX hermaphrodite injected with *sup-7* tRNA. The pseudomale possesses a vestigial male tail (white arrowhead); the hermaphrodite has a whip-like tail (arrow). The pseudomale either has no vulva, as shown here, or a grossly malformed vulva; the hermaphrodite has a normal vulva (small black arrowhead). The pseudomale gonad is intersexual; the rescued gonad is indistinguishable from a hermaphrodite gonad, except that oocytes are made later than normal. Only one hermaphrodite has laid eggs, so some defect in the egg-laying system is probable. The injected animal carries a mutation in *dpy-4* (*e1166*) which is closely linked to *tra-3* on chromosome IV; this distinguishes the *tra-3* homozygote among progeny of a heterozygous parent. In addition, the animal is homozygous for a mutation in *nuc-1* (*e1392*), which encodes the major endodeoxyribonuclease.

homozygous *tra-3* animal is particularly striking as no hermaphrodites were observed among the progeny of control animals (see below). Thus, 60% of the injected animals showed the effects of suppression. It is unclear, however, whether the injected RNA acts before or after fertilization. The genotype of the injected animals was confirmed by their production of pseudomales (expected to arise from the uninjected half gonad). The genotype of the rescued hermaphrodite progeny produced after injection was verified in turn by examining their self progeny; in all cases, only *tra-3* pseudomales were produced, confirming that these phenotypic hermaphrodites were homozygous *tra-3* animals.

As controls, *sup-7* tRNA was injected into animals homozygous for the non-amber allele, *e1767*, and wild-type tRNA was injected into animals homozygous for the amber allele, *e1107*. In addition, 4,000 progeny of uninjected *e1107/e1107* hermaphrodites were examined. In both injected and uninjected control animals, no hermaphrodite progeny were produced (Table 1). Therefore, production of hermaphrodites after injection of *sup-7* tRNA into *e1107/e1107* animals must result from *in vivo* suppression.

The small number of hermaphrodites made by each injected parent may result from the relative impurity of the tRNA injected, or it may mean that the injected molecules are concentrated into a few oocytes. Experiments are in progress to determine more precisely when the rescued embryos are made after injection, and what the effects of injecting a more purified tRNA preparation might be on the efficiency of suppression.

In vivo suppression by *sup-7* tRNA provides a convenient means of assaying and manipulating gene function during embryogenesis. Injection of *sup-7* tRNA into individual blastomeres after fertilization should generate precisely engineered genetic mosaics for subsequent analysis of tissue-specific developmental effects. In addition, suppression of *tra-3* or other genes with suppressible alleles may prove useful as a bioassay to detect transcripts of a cloned DNA encoding the *sup-7* gene *in vivo*.

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When do carcinogen-treated 10T1/2 cells acquire the commitment to form transformed foci?

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A major advance in analysing the mechanisms of chemical carcinogenesis has been the development of cell culture systems in which normal rodent cells can be transformed as a result of *in vitro* exposure to specific chemicals or radiation¹⁻⁶. An unexpected finding in these systems is that the frequency of transformation is often much greater than that of induction of drug-resistant mutants^{3,6-10}. In addition, studies using the technique of cell spreading have suggested that the latent period between exposure of cell cultures to carcinogens and the emergence of cells having the heritable ability to form transformed foci is also much greater than that for the induction of drug-resistant mutants^{8,11,12}. In most of the above transformation studies, the cells were exposed to carcinogens at low cell densities and, therefore, mutation-like events that might be induced with a low frequency could have been missed. Thus, we have now analysed the kinetics of cell transformation of C3H 10T1/2 cells when exposed to the potent ultimate carcinogen ($\pm$)7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) in conditions of high cell density. We report here that in these conditions acquisition of the ability to form transformed foci and acquisition of ouabain resistance occur with similar kinetics and within 2 days of carcinogen exposure.

To determine when carcinogen-treated C3H 10T1/2 cells acquire the heritable property of ouabain resistance or the ability to form morphologically transformed foci we used the protocol described in Fig. 1 legend. Replicate plates of cells were trypsinized and replated at the same density at various times after exposure to the carcinogen, and then the secondary plates were incubated and scored for the number of ouabain-resistant clones or the number of foci of transformed cells. The rationale is that if colonies of committed cells have already begun to form on the donor plates, then the individual cells of such colonies will be dispersed on the secondary plates where they will form multiple colonies or foci. Thus, the time after carcinogen treatment at which reseeding expands the number of colonies or foci indicates, approximately, the time at which the treated cells have become committed or clonogenic for the property being measured. Since the plating efficiency during the replating is < 100%, the number of colonies or foci on the secondary dishes may actually be a lower limit of the number of committed cells. We used the same initial cell density (50,000 cells per 6 cm plate) to study the induction by BPDE of both ouabain resistance and transformation.

Figure 1a shows representative plates from BPDE-treated and control cultures that were used to select for ouabain-resistant mutants, with or without the replating procedure. It is obvious that replating markedly increases the number of ouabain-resistant colonies. This effect was apparent within 2 days of BPDE treatment and reached a plateau within 5 days, at which time the cells on the primary plates were confluent (Fig. 2a). The replating procedure led to about an eightfold increase in the number of colonies growing in the presence of ouabain. This increase was less than the 16-fold increase one would expect if the cells had undergone approximately four doublings before reaching confluency. This difference may be

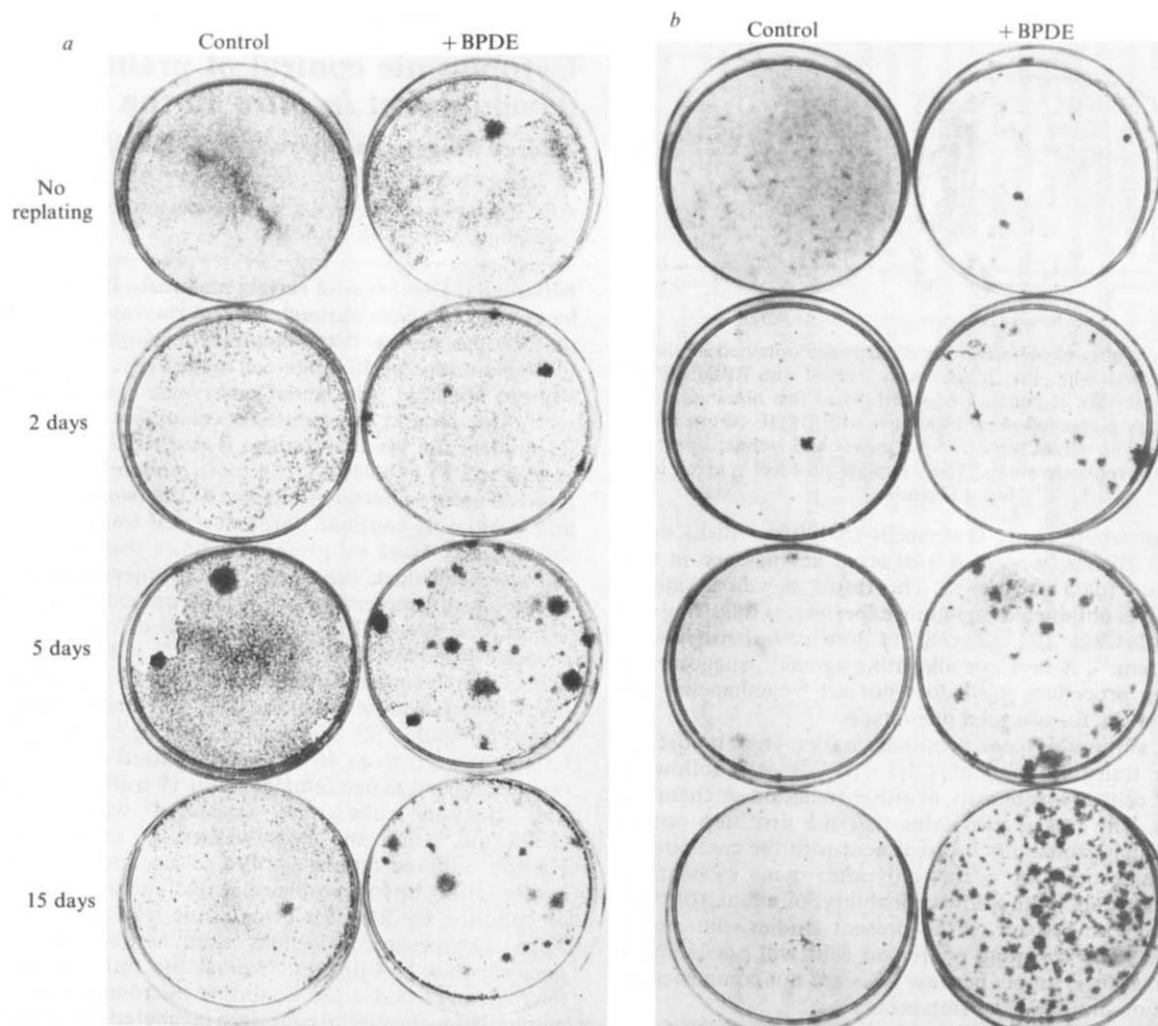


Fig. 1 Effect of replating on the number of ouabain-resistant colonies and the number of transformed foci. *a*, Representative plates of cultures replated at various times after BPDE treatment and stained for ouabain-resistant colonies. *b*, Representative plates stained for transformed foci. C3H 10T1/2 Cl8 (ref. 15) was provided by Dr J. Bertram and grown on plastic tissue culture plates (Nunc, Denmark) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (GIBCO). 50,000 cells were seeded per 6 cm plate; 24 h later the plates were rinsed with phosphate-buffered saline (PBS) and incubated for 1 h with 2 ml of DMEM containing either BPDE (50 ng ml⁻¹) in 0.01% of tetrahydrofuran (THF) or 0.01% THF (solvent control). The BPDE stock solution in THF was diluted into DMEM immediately before use. After incubation, the plates were rinsed with PBS and 5 ml of DMEM supplemented with 5% calf serum (DMEM-5) were added to each plate; this medium was changed every week. Four to six weeks after the cells reached confluency, either on the primary or secondary plates (see below), they were fixed with 3.7% formaldehyde, stained with Giemsa and the sum of type II and type III foci was determined as previously described¹⁵. For the ouabain resistance mutation assay, the protocol was similar except that DMEM-5 containing 3 mM ouabain was added to the dishes either 62 h after treatment with BPDE or THF, or 24 h after the cells were reseeded onto the secondary plates. Colonies were fixed, stained and counted 2–3 weeks after exposure to ouabain¹³. For the replating studies, at various times after the cells were treated with BPDE, or THF, five replicate plates were dissociated with 0.25% trypsin/versene, the cells were pooled in 25 ml of DMEM-5 and seeded onto five new Petri plates. Thus the densities of cells on the secondary plates were approximately the same as that on the donor primary plates.

due to the known decrease in the number of ouabain-resistant mutants recovered if the expression time (time between treatment and exposure to ouabain) is delayed for several days^{13,14}.

Figure 1b shows representative plates from parallel studies in which BPDE-treated and control cultures were or were not replated at various times after treatment and the plates scored for foci of transformed cells 4–6 weeks after the cells had reached confluency. It is obvious that the replating procedure markedly increased the number of transformed foci that were detected. This increase was apparent within 48 h of BPDE treatment and reached a plateau between 8 and 12 days (Fig. 2b). At this plateau the replating procedure led to about a 16-fold increase in the number of foci, which one would expect if the focus-forming cells had undergone about four doublings before replating.

Three additional experiments using the protocol described in Fig. 1 legend gave results that were qualitatively similar to those shown in Fig. 2a, b. It is of interest that the kinetics of

induction of transformed cells by BPDE were quite similar to those for induction of ouabain-resistant mutants, suggesting that both processes involve similar events. Although the cells on the primary plates became confluent by 5 days after the BPDE treatment, the number of transformed foci observed after replating continued to rise from days 5 to 15 (Figs 1b, 2b). We presume that this is because the cells in the process of transformation continue to replicate on the primary plates, even after the rest of the cell population ceases proliferating. Growth curves revealed that the concentration of BPDE (50 ng ml⁻¹) used in these studies produced negligible cytotoxicity (data not shown).

Thus, it seems that within 48 h of BPDE treatment, variants emerge that have a heritable alteration in both ouabain resistance and morphological transformation. As the doubling time of these cells is 18–24 h (ref. 15), this commitment occurred within one or two doublings after BPDE treatment. In similar experiments using BPDE and replating at the same time

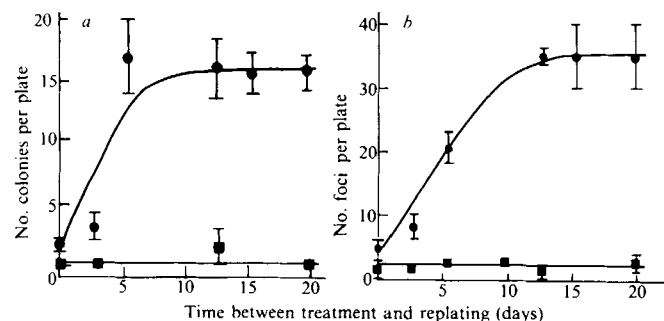


Fig. 2 *a*, Number of ouabain-resistant colonies obtained on the secondary plates when the cultures were treated with BPDE (●) or the solvent (■). *b*, Number of transformed foci obtained on the secondary plates following treatment with BPDE (●) or the solvent (■). The values represent the means and standard deviations of five replicate plates. The complete protocol is given in Fig. 1 legend.

intervals, but where many fewer cells (5,000 per dish) were treated with BPDE, we did not observe an increase in the number of foci (data not shown). This result, as well as similar negative results obtained in replating experiments following the treatment of C3H 10T1/2 cells at low cell density with benzo(a)pyrene¹¹, X rays⁸, or alkylating agents¹², suggest that the replating procedure itself does not act by enhancing the acquisition of the transformed phenotype.

Previous authors^{8,9} have proposed a two-step model to explain the transformation of C3H 10T1/2 cells following exposure of cells at low density to either radiation or chemical carcinogens. The model postulates that the first step occurs with a very high probability on treatment with the carcinogen, and that the second step occurs only after many subsequent cell generations and with a low probability, of about 10^{-6} per generation⁹. With respect to the present studies, the model predicts that early replating of treated cells will not increase the number of foci, simply because cells are not committed to transformation until this late step occurs.

Our observation of an early commitment of cells to form transformed foci when the initial exposure to carcinogen occurs at a high cell density, but not at a low cell density, might be explained by assuming that a high cell density increases the likelihood that the second step occurs more rapidly. Alternatively, it is possible that transformation can also occur via a single mutation-like event, but that this occurs with a low probability, in the range of 10^{-4} to 10^{-5} . Thus this route would be observed only when one exposes about 10^4 or more cells to the carcinogen. If two different routes for cell transformation do exist, then it will be of interest to determine which of them involves random point mutations, genomic rearrangements or epigenetic mechanisms, and whether or not such changes involve protooncogenes or long terminal repeat sequences normally present in the mouse genome^{10,16}. Regardless of the mechanism, the replating procedure described in the present study may be of practical significance because it shortens the lag period and increases the sensitivity of *in vitro* assays for carcinogens in the C3H 10T1/2 cell system.

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Cytoplasmic control of preimplantation development *in vitro* in the mouse

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Although the embryos of certain mammals, including man, can be cultured *in vitro* through several cleavage divisions^{1,2}, it is only in the mouse that complete development through the preimplantation period (one-cell to the blastocyst stage³) occurs without affecting subsequent embryonic viability after transfer^{1,4}. But even in the mouse, development *in vitro* from the one-cell to the blastocyst stage is restricted to certain inbred strains and F_1 hybrids^{1,3,5,6}. In most randomly bred strains the one-cell embryo becomes blocked at the two-cell stage *in vitro* and it will only continue development if transferred back into the oviduct⁷. Here we present evidence that this so-called 'in vitro two-cell block' can be obviated by injecting small amounts of cytoplasm from embryos which do not exhibit this block.

Fertilized one-cell eggs were obtained from superovulated randomly bred MF1 (OLAC, UK) and F_1 (C57BL/6 × CBA/H) hybrid females mated with MF1 males. Intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG; Organon) and 5 IU human chorionic gonadotropin (HCG; Organon) were given 48 h apart. Fertilized one-cell eggs were recovered from mated females about 19 h after HCG injection. The cumulus cells were dispersed with hyaluronidase (150 U ml^{-1}) and the eggs washed in several changes of HEPES-buffered mouse embryo culture medium, M2 (ref. 8) before setting up for cytoplasmic injections at the one-cell stage or culturing for 24 h for cytoplasmic transfer at the two-cell stage. Cytoplasmic injections were carried out using glass micropipettes ($\sim 4 \mu\text{m}$ diameter at the tip) attached to Agla microsyringes and a De Fonbrune micromanipulator. Before manipulation, groups of eggs were incubated for 15 min at 37°C in mouse embryo culture medium no. 16 (ref. 9) containing cytochalasin D (Sigma) at a concentration of between 0.2 and $0.5 \mu\text{g ml}^{-1}$ (ref. 10). To maintain a stable pH (between 7.2 and 7.4) in air for microinjections, the eggs were transferred to 50–100- μl drops of M2 medium plus cytochalasin D overlaid with paraffin oil, in plastic culture dishes (Falcon). Although exposure of eggs to cytochalasin D during micromanipulation facilitated egg penetration and withdrawal and delivery of the cytoplasm, preliminary experiments showed that the period when such manipulations could be accomplished without affecting the subsequent survival of the egg was critical: between 15 and 45 min after placing eggs in cytochalasin D (our unpublished observations). Care was taken not to withdraw pronuclei or blastomere nuclei when removing cytoplasm from donor eggs. After injection, the eggs were washed and cultured in medium 16. The eggs were observed several hours after incubation to determine the numbers surviving microinjection. Subsequent observations were made every 24 h until development of control embryos had reached the blastocyst stage.

In the first series of experiments (Table 1) cytoplasm from fertilized one-cell F_1 (C57BL/6 × CBA/H) eggs was injected into one-cell fertilized eggs of the randomly bred MF1 strain ($\sim 22 \text{ pl}$ of cytoplasm per egg). Over 50% of the eggs survived the injection and some of the eggs seemed to be proceeding further in preimplantation development compared with the uninjected MF1 controls. The injection of equivalent volumes of culture medium had no effect on further development of either strain (our unpublished observations). The inability of uninjected fertilized one-cell MF1 eggs to complete preim-

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Table 1 *In vitro* development of fertilized one-cell MF1 eggs after injection of cytoplasm from fertilized one-cell F₁ (C57BL/6×CBA/H) eggs

No. of 1-cell eggs injected	No. surviving (%)	No. cleaved (%)	2-Cell	Developmental stage reached during 96 h in culture			Blastocyst
				4-Cell	8-Cell	Morula	
105	58 (55)	46 (79)	35	2	4	5	—
Uninjected controls							
	No. of 1-cell eggs						
MF1	43	39 (91)	37	1	1	—	—
F ₁	45	45 (100)	6	1	1	2	35

plantation development *in vitro* confirms all previously reported findings¹.

A second series of experiments was designed to examine the effect of injecting F₁ cytoplasm from two-cell embryos into MF1 eggs at the two-cell stage. For these purposes both F₁ and MF1 two-cell eggs were grown from the one-cell stage *in vitro*. Only one blastomere was injected with cytoplasm (~8 pl), thus the remaining uninjected blastomere provided a within-embryo control. About 70% of the MF1 eggs receiving cytoplasm from F₁ two-cell eggs developed to the morula and blastocyst stages during 72 h in culture but none of the MF1 eggs receiving MF1 cytoplasm developed beyond the four-cell stage (Table 2 and Fig. 1). In most of the MF1 eggs injected with F₁ cytoplasm (~60%) both the uninjected and injected blastomeres divided at the second cleavage division. As the injections were performed when the two-cell embryo was about midway between the first and second cleavages, the cytoplasmic bridge was probably still present, allowing material to pass between the two blastomeres as shown by Lo and Gilula¹¹. A single MF1 egg developed to the blastocyst stage in the uninjected control group, confirming previous observations¹ where a small propor-

tion of randomly bred embryos have been shown to complete preimplantation development from the one-cell stage *in vitro*.

Although the *in vitro* arrest observed in the development of zygotes from certain mouse strains may be due to some artefact of the culture system, our results clearly demonstrate that this block can be overcome by injecting small quantities of cytoplasm (~8 pl—about 4% of the egg volume at the two-cell stage) from two-cell eggs fully capable of completing preimplantation development *in vitro*. The component(s) in the F₁ cytoplasm responsible for initiating further development of the MF1 zygotes *in vitro* are unknown. It is apparent that cytoplasm from the two-cell stage eggs is better able to promote further development *in vitro* than that from one-cell stage eggs. There were marked differences in the nature of the cytoplasm at the one- and two-cell stages after exposure to cytochalasin D (one-cell stage cytoplasm dispersed freely in the culture medium but at the two-cell stage the cytoplasm remained compacted and sausage-shaped) suggesting changes in its structural organization (our unpublished observations). It is possible that either the components added in the cytoplasm injected at the one-cell stage are leached out during culture to the two-cell stage or

Fig. 1 *a*, Two-cell MF1 embryos (grown from the one-cell stage *in vitro*) shortly after injecting F₁ cytoplasm into one-blastomere. *b*, Development of injected two-cell MF1 embryos after a further 24 h incubation *in vitro*. Note the different types of development occurring. The degenerated blastomeres are those which did not receive an injection of cytoplasm. *c*, Uninjected MF1 controls still at the two-cell stage 24 h later. *d*, Injected two-cell MF1 embryos 48 h after injection. These are at the four-cell, morulae and compacted morulae stages of development. *e*, Various developmental stages consisting of compacted morulae, blastocysts and expanded blastocysts, 72 h after injection—that is, after a total of 96 h *in vitro*. Note the oil droplet in one blastomere of the middle embryo on the left. This was injected with cytoplasm into one of the blastomeres at the two-cell stage. *f*, Uninjected MF1 controls after a similar 96-h culture period remain at the two-cell stage of development. ×125.

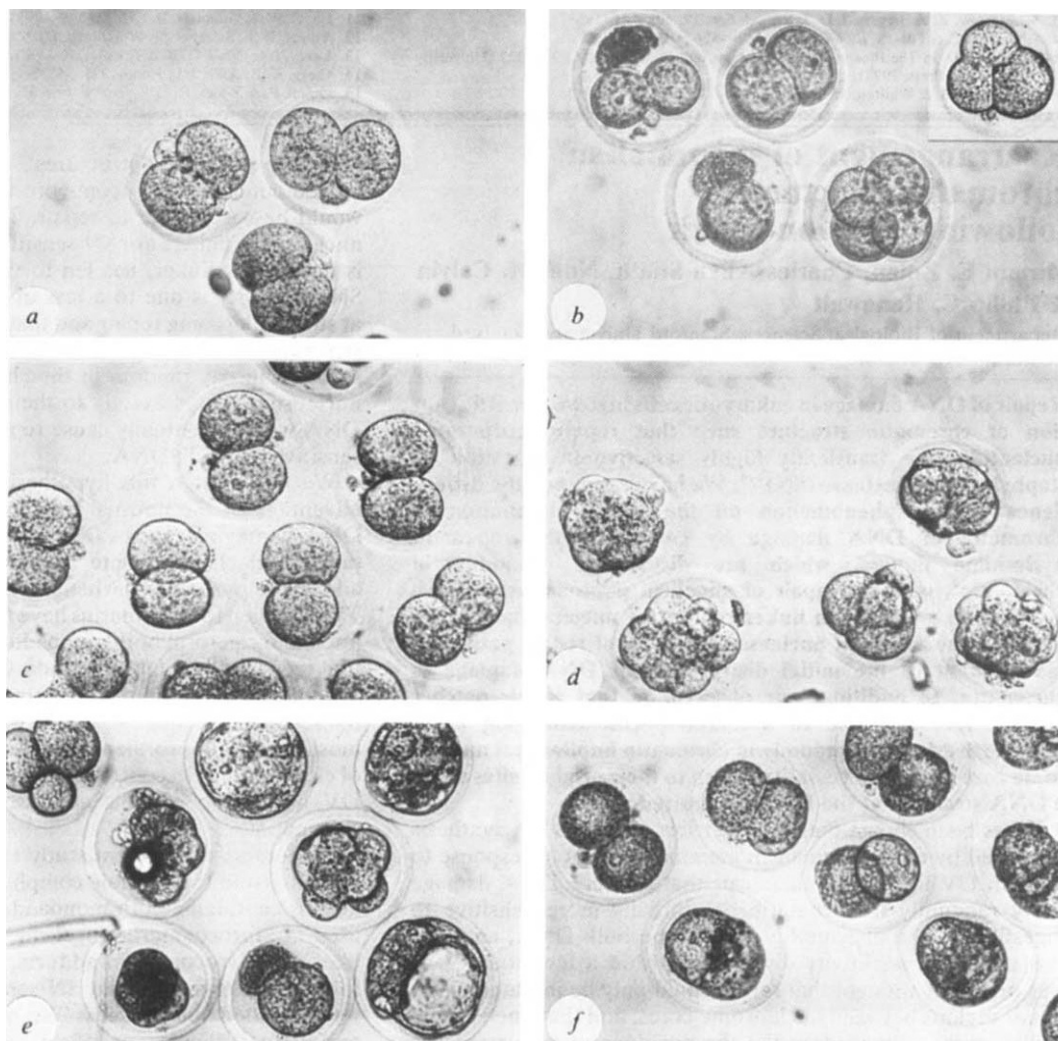


Table 2 *In vitro* development of one-cell MF1 eggs after injection at the two-cell stage with cytoplasm from two-cell stage eggs

Source of cytoplasm	No. of 2-cell eggs injected	No. surviving (%)	Developmental stage after 24 h			Developmental stage reached during subsequent culture for 48 h					
			2-Cell	3-Cell	4-Cell	2-Cell	3-Cell	4-Cell	8-Cell	Morula	Blastocyst
F ₁ hybrid	72	42 (58)	9	8	25	9	2	1	—	13	17
MF1	24	18 (75)	10	6	2	10	6	2	—	—	—
Uninjected controls											
	No. of 1-cell eggs										
MF1	92		79	—	13	79	—	12	—	—	1
F ₁	73		1	—	72	1	—	4	5	4	59

Development *in vitro* of fertilized one-cell MF1 eggs when one of the blastomeres at the two-cell stage was injected with cytoplasm from either two-cell F₁ hybrid (C57BL/6 × CBA/H) or two-cell MF1 embryos cultured from the one-cell stage. Both injected and uninjected eggs were grown from the one-cell stage *in vitro*.

the quantity of substance(s) injected is inadequate to overcome the block. Alternatively, new vital components may be present in the cytoplasm at the two-cell stage arising either by transcription from the embryonic genome^{12,13} or by synthesis from maternal messenger RNA¹⁴. Perhaps these processes are not fully initiated during the culture of zygotes from certain strains.

The differences between *in vitro* development of one-cell eggs from various strains may have a genetic basis. Subtle differences in the amount of stored maternal mRNA, the activity of enzymes, the activity of the mitochondria and the structure and permeability characteristics of the egg plasma membrane may all contribute to the successful development of the one-cell embryo *in vitro*. Higher levels of ATP have been found in F₁ hybrid two-cell eggs grown from the one-cell stage *in vitro* compared with similar eggs from a randomly bred

strain¹⁵. Regulation of expression from the embryonic genome may well be controlled by factors in the egg cytoplasm and further studies with nuclear transfers might solve this problem.

Whatever the reasons for the inability of some one-cell mouse embryos to complete preimplantation development *in vitro*, the evidence so far shows that special environmental conditions are required to obtain early development *in vitro*, and these are still unknown for the eggs and embryos of most mammals. Until techniques are available for direct studies of mammalian eggs and embryos *in vivo*, our knowledge of the requirements of these early stages of development depends on biochemical analysis of eggs, embryos and oviduct secretions.

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Rearrangement of mammalian chromatin structure following excision repair

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Repair of DNA damage in eukaryotic cells involves local disruption of chromatin structure such that repair-incorporated nucleotides are transiently highly sensitive to digestion by staphylococcal nuclease (SN)¹⁻⁴. We have examined the dependence of this phenomenon on the initial distribution in chromatin of DNA damage by comparing the repair of pyrimidine dimers, which are distributed randomly in chromatin⁵, with the repair of angelicin photoadducts, which are formed primarily in linker regions of nucleosomes^{6,7}. We find that the transient nuclease sensitivity of repair patches is independent of the initial distribution of DNA damage in chromatin. In addition, our observation that repair patches produced in response to a linker-specific damaging agent become distributed randomly in chromatin implies that nucleosome cores do not necessarily return to their original sites along a DNA strand after the DNA is repaired.

It has been shown that the stretches of new DNA synthesis produced by excision repair in mammalian cells in response to 254 nm UV light or to chemicals that produce DNA damage fairly randomly in chromatin are initially more sensitive to digestion by the enzyme SN than is the bulk DNA, and that this enhanced sensitivity disappears within a few hours¹⁻⁴. It was originally thought that repair could only be initiated in the linker regions between nucleosome cores, and that nucleosome sliding eventually randomized the positions of repair patches

with respect to core structures¹. Remaining lesions would be moved concomitantly from core to linker regions, where they would be susceptible to repair. The realization that although nucleosome linkers are SN-sensitive, not all SN-sensitive DNA is necessarily linker, has led to the hypothesis that the initial SN sensitivity is due to a loss of native nucleosome structure at sites undergoing repair and that complete restoration of such structures requires several hours^{3,4}. In cases where the damaged sites are nearly random in the chromatin, such restoration of nucleosome cores exactly to their original positions along the DNA would eventually cause repair patches to exhibit the SN sensitivity of bulk DNA.

We have tested this hypothesis more rigorously by taking advantage of the unusual properties of the furocoumarins as DNA-damaging agents. These are conjugated tricyclic compounds which intercalate into DNA and can form covalent adducts to pyrimidines when activated by 360 nm light (UVA). The psoralen furocoumarins have their rings in a linear configuration and can form both monoadducts and bifunctional adducts that cross-link the DNA strands. Other furocoumarins, such as angelicin, have rings in an angular configuration and form only monoadducts in the conditions we have used^{8,9}. It seems that most, if not all, psoralen adducts are formed in linker regions of chromatin^{6,7}. In contrast, the pyrimidine dimers formed after UV irradiation of cells are distributed nearly randomly in chromatin^{5,10}.

To confine the present study to the repair of monoadducts so as to avoid the possible complications of interpreting results in cells containing both monoadducts and cross-links, we have used the furocoumarin angelicin. Although we expected that, like other furocoumarin adducts, those of angelicin would be formed predominantly in SN-sensitive regions of chromatin, we examined this directly. We observed that, in contrast to pyrimidine dimers, angelicin adducts in chromatin were

released by SN with kinetics consistent with their being primarily in SN-sensitive linker DNA (Fig. 1).

We compared the repair of angelicin adducts with that of pyrimidine dimers in human cell line T98G (refs 9, 11) to determine how the distribution of DNA damage in chromatin affects repair replication. The time course for repair synthesis is the same after UV damage as after treatment of cells with angelicin and UVA (Fig. 2). Thus, damage in linker DNA is apparently recognized and repaired with the same kinetics as damage that is randomly distributed in chromatin. This result has also been obtained with human diploid fibroblasts⁹ and African green monkey kidney cells¹².

We also examined the SN sensitivity of DNA synthesized during excision repair of UV or angelicin damage. In both cases, the repair patches were initially more sensitive than the bulk chromatin, and this difference was considerably reduced after a further 24 h incubation of the cells (Fig. 3).

The measurement of the relative amounts of radioisotope incorporated by semi-conservative replication and by repair synthesis is important for interpretation of these data. Even though our experiments were performed on confluent, contact-inhibited cultures, some radioactive precursor was incorporated

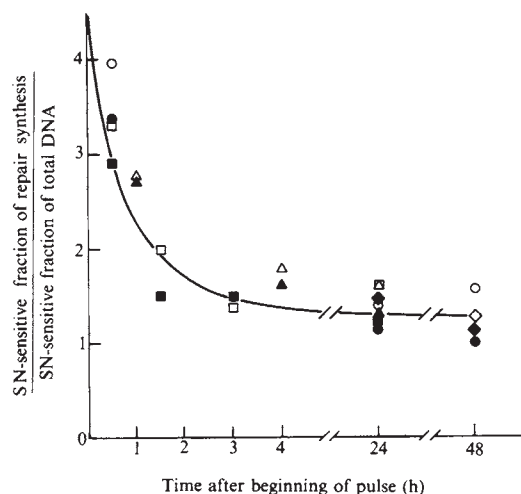


Fig. 1 Sensitivity of angelicin adducts or pyrimidine dimers in chromatin to digestion by SN. Cells were grown for 15 h in ^3H -deoxythymidine ($10 \mu\text{g ml}^{-1}$) and treated with $25 \mu\text{g ml}^{-1}$ angelicin and 7.5 kJ m^{-2} UVA. Nuclei were prepared and either digested with SN as described in Fig. 3 legend, or held on ice. After aliquots had been removed for determination of the extent of digestion, the nuclei were lysed and treated with proteinase K. DNA was extracted with phenol and then chloroform/isoamyl alcohol and precipitated with ethanol. A portion of the purified DNA from nuclei not digested with SN was then treated with SN, the extent of digestion analysed as before and the DNA reprecipitated as above. For analysis of adduct frequencies, the DNAs were digested to mononucleotides with a mixture of DNase II and spleen phosphodiesterase. These digests were analysed by electrophoresis through $0.3 \times 10 \text{ cm}$ tube gels of 30% acrylamide/3% bis-acrylamide, in a continuous-flow collection apparatus. Samples collected from the bottom of the gel were assayed for radioactivity by scintillation spectrometry. The positions of angelicin adducts were determined from experiments with ^3H -labelled angelicin and pure DNA labelled with either ^{32}P or with ^{14}C -thymine. These experiments showed that nearly all adducts were to thymine, which allowed us to calculate adduct frequencies as the fraction of radioactivity loaded onto the gel that appeared in these positions. The per cent adducts remaining was calculated from these frequencies and the fraction of DNA digested. Random distribution of adducts in chromatin would yield the theoretical result shown by the dashed line. ●, Digestion of nuclei from angelicin-treated cells. ○, Digestion of purified DNA from nuclei not treated with SN. For pyrimidine dimers (×), cells were grown in ^3H -deoxythymidine and irradiated with 10 J m^{-2} UV. Nuclei were prepared and digested with SN, then lysed, treated with proteinase K and the DNA was centrifuged through a neutral sucrose gradient in an SW60 rotor in conditions that placed a 175-base pair marker DNA in the centre of the tube. The profile obtained by assaying total radioactivity in aliquots of the fractions allowed both the calculation of the fraction of DNA digested and isolation of the undigested DNA, which was further purified by centrifugation in CsCl gradients. A sample of DNA from nuclei not digested with SN was also prepared by centrifugation in CsCl. The DNAs so obtained were analysed for pyrimidine dimer content by TLC after acid hydrolysis²². In these experiments, the actual adduct frequencies were roughly comparable; the conditions used produced about 25 pyrimidine dimers and about 50 angelicin adducts per 10^8 daltons DNA.

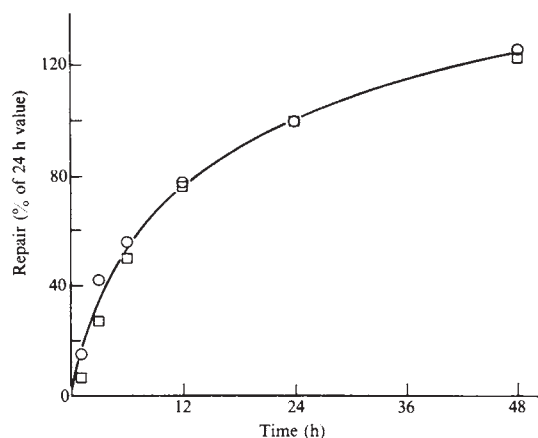


Fig. 2 Time course of the repair replication in T98G cells in response to 10 J m^{-2} UV (○) or $25 \mu\text{g ml}^{-1}$ angelicin and 7.5 kJ m^{-2} UVA (□). Treatment of cells was as in ref. 9 except that DNA-damaging treatments were done at 4°C . Repair synthesis was measured as described in ref. 23. In these conditions, the actual repair synthesis observed per unit DNA for angelicin was about 60% of that with UV.

into DNA synthesized by semi-conservative replication. The radioisotope so incorporated had the same SN sensitivity as bulk chromatin in these digestion conditions (ref. 13 and M.E.Z., unpublished observation). The amount of residual semi-conservative replication in a culture depended on the dose and type of damage, and the condition of the culture, which varied from experiment to experiment. In our experiments, DNA synthesis was always almost completely inhibited by the angelicin and UVA treatment used, but after 10 J m^{-2} UV as much as 30% of the isotope incorporated during a 30 min labelling period was in DNA made by semi-conservative replication. Thus, the apparent increase in the SN sensitivity of repair label relative to the prelabel was smaller after UV damage than after angelicin damage (Fig. 3). To compare experiments done at different times and using different damaging agents, it is necessary to normalize SN digestion data in a manner that corrects for the amount of isotope incorporated into DNA by semi-conservative synthesis. Such a method was developed by Smerdon and co-workers¹³. In their analysis, which has been discussed in detail^{4,13}, the ratio of the fraction of repair label that is SN-sensitive to the fraction of the total genome that is SN-sensitive is determined at various times after a short labelling period.

We analysed the data from several experiments by this method (Fig. 4). In each experiment, we measured directly the amounts of isotope incorporated by replication and repair; some of the cells were incubated with ^3H -bromodeoxyuridine and the DNA synthesized by semi-conservative replication was resolved from parental-density DNA containing repair patches by CsCl density gradient centrifugation as described in ref. 4.

Our analysis showed that the relative SN sensitivity of repair patches was initially high and decreased with time in the same manner after UV or angelicin damage (Fig. 4). The fact that the magnitude of the initial SN sensitivity of repair patches is about the same for repair of the two different adducts does not address the question of the cause of this increased nuclease sensitivity. It merely confirms the notion that all repair patches are initially nuclease-sensitive⁴, whether because they were initiated in linker DNA or because the repair process itself requires dissolution of normal nucleosome structure. However, our observation that the repair patches produced in response to angelicin damage become distributed nearly randomly in chromatin with time indicates that the final distribution of repair patches in chromatin is not the same as the initial, linker-specific distribution of this DNA damage. We have shown previously that the size of repair patches synthesized in response to angelicin and UV damage is about 25 nucleotides⁹, slightly smaller than the average size of nucleosome linkers in human cells¹⁴. A large fraction of the nucleotides in repair patches

made during repair of angelicin damage would remain in linker DNA (and would therefore be SN-sensitive) if nucleosomes re-formed at their original positions after DNA is repaired. However, as repair patches made in response to angelicin damage eventually exhibit the SN sensitivity characteristic of

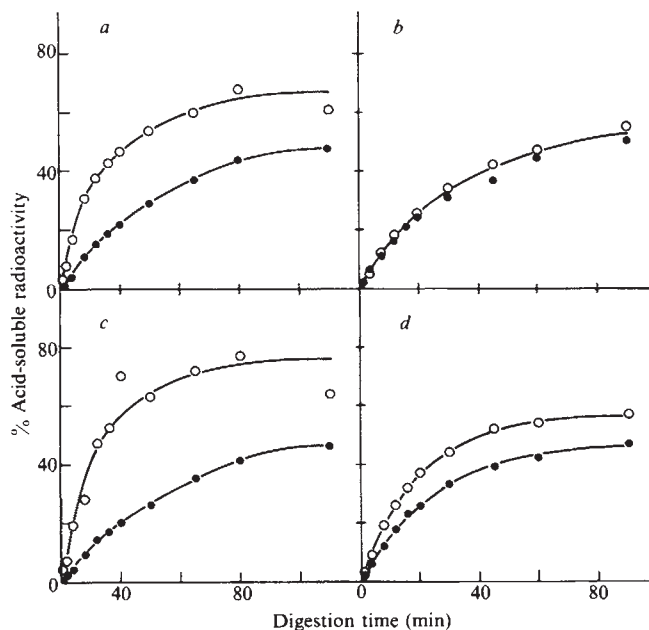


Fig. 3 Digestion of repair-labelled nuclei with SN. T98G cells were treated with UV (a, b) or angelicin and UVA (c, d) as described in Fig. 1 legend and incubated at 37°C for 30 min in conditioned medium containing 10 μ Ci ml⁻¹ ³H-deoxythymidine (50 Ci mmol⁻¹; NEN). This medium was removed and nuclei were prepared immediately (a, c) or after the cells were incubated for 24 h in medium containing 100 μ M non-radioactive deoxythymidine (b, d). To prepare nuclei, cells were first washed twice with 10 mM Tris, pH 8, 1 mM CaCl₂. To each dish was added 3 ml of this buffer and the dishes were held on ice for 20 min. The buffer was vigorously pipetted several times against the cell monolayer with a Pasteur pipette to release nuclei which were then pelleted by centrifugation at 2,000 r.p.m. for 10 min in an IEC universal centrifuge. The pellet was washed twice in 10 mM Tris, pH 8, 1 mM CaCl₂, 0.25 M sucrose, and resuspended in this buffer at a concentration equivalent to 180 μ g ml⁻¹ DNA. Samples were incubated at 37°C for 5 min and SN (Millipore) was added to 8 U ml⁻¹. At the times shown, 0.1-ml samples were taken and added to an equal volume of ice-cold 2 M NaCl, 2 M perchloric acid²⁴. After 10 min on ice, samples were centrifuged for 5 min in a Beckman microfuge B, and 0.15 ml of each supernatant was assayed for radioactivity in 1 ml H₂O plus 10 ml of a 1:2 mixture of Triton/Omnifluor (NEN). Aliquots of undigested nuclei were assayed for total radioactivity incorporated as described in ref. 13. ○, ³H repair label; ●, ³²P prelabel.

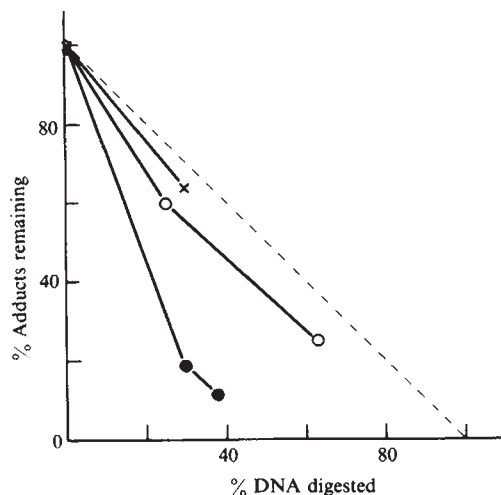


Fig. 4 Change with time of the enhanced SN sensitivity of repair patches synthesized in the first 30 min after treatment of cells with UV (solid symbols) or angelicin and UVA (open symbols). Different symbols represent different experiments. The analysis used was that described by Smerdon *et al.*¹³, except that we measured the relative amounts of radioactivity incorporated by semi-conservative replication and repair as described in the text. The experimental details are described in Fig. 3 legend.

a random distribution in chromatin, we conclude that nucleosome cores do not obligatorily re-form at their original positions after DNA is repaired. Our observations support models of random location of nucleosomes with respect to DNA sequences¹⁵⁻¹⁷, but they are not sensitive enough to rule out the possibility that nucleosome cores do occupy preferred sites in some regions of the genome¹⁸⁻²¹.

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Selective reinnervation of adult mammalian muscle by axons from different segmental levels

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The ability of axons to innervate selectively subsets of a population of largely similar target cells is undoubtedly important for the establishment of ordered patterns of neural connections. Vertebrate skeletal muscle is well suited for studies of factors that account for selective synapse formation as a wealth of information is available about the structure, function and molecular components of its neuromuscular junctions. Furthermore, skeletal muscle is readily reinnervated following nerve damage, permitting study of synaptogenesis in an accessible postembryonic environment. However, although selective synapse formation clearly occurs elsewhere in the adult peripheral nervous system¹⁻¹⁰, previous attempts to demonstrate selectivity in the reinnervation of mammalian muscles have been disappointing⁷⁻¹⁵. We have now used a novel approach to reinvestigate this issue, and describe here a situation in which two different adult rat muscles are preferentially reinnervated by axons from different spinal segments. Our results suggest that adult muscles differ in some stable quality, perhaps related to their position, which influences the innervation they accept.

Our approach was based on studies of the segmental innervation of sympathetic ganglion cells by autonomic preganglionic axons. The cervical sympathetic trunk, which innervates the

superior cervical ganglion (SCG; Fig. 1), contains populations of preganglionic axons that arise from several segments of the spinal cord^{1-3,16}; axons from each segment can be stimulated separately via the appropriate ventral root¹⁷. Preganglionic axons from different segments preferentially reinnervate different classes of neurones in the SCG^{1,2,7}. In particular, Purves *et al.*³ have shown that ganglia transplanted from the fifth thoracic (T5) level to the neck are reinnervated by a more caudal subset of cervical trunk axons than are reimplanted SCGs. Thus, ganglia from different levels may differ in some quality that biases synapse formation. As sympathetic preganglionic axons can be made to innervate skeletal muscle¹⁸⁻²⁰, and muscles survive free grafting²¹, we were able to investigate whether muscles from different segments are also differentially reinnervated by subpopulations of preganglionic axons.

The muscles we used, the external intercostals, are a set of 12 similar muscles in the thorax, each of which joins two adjacent ribs and is innervated by axons from the corresponding spinal segment²². Pieces of external intercostal muscle were transplanted from either the second (T2) or the eighth (T8) thoracic interspace to the neck of the same rat; the SCG was removed and the proximal cut end of the cervical trunk attached to the implant (Fig. 1). Two to fourteen weeks later, the transplanted muscle, cervical trunk, sympathetic chain and ventral roots were dissected in continuity and mounted in a bath^{2,3}. Individual ventral roots were stimulated with suction electrodes and synaptic potentials were recorded intracellularly from

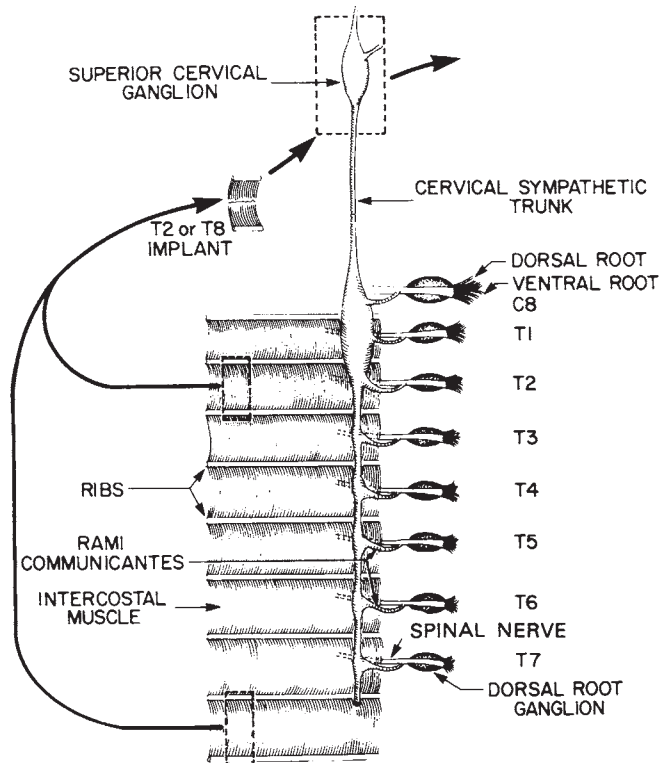


Fig. 1 External intercostal muscles from the second (T2) or eighth (T8) thoracic level were transplanted to the neck where they were reinnervated by axons of the cervical sympathetic trunk. Adult, male rats (Sprague-Dawley; 150–200 g) were anaesthetized with chloral hydrate, and a 2–3 mm wide piece of either the T2 (with second and third ribs attached) or the T8 (with eighth and ninth ribs) intercostal muscle removed. The internal intercostal muscle was peeled away, leaving a sheet of predominantly intact external intercostal muscle fibres attached to ribs at both ends. An incision was then made in the neck and the SCG removed. The external intercostal muscle was sutured by its ribs to the superficial surface of the omohyoid muscle and the cut end of the sympathetic trunk pulled through a small hole near the edge of the implant. For simplicity, muscle and nerve are shown on the same side; in reality, muscle was taken from the left side and transplanted to the right, to avoid inadvertent damage to the sympathetic trunk or ventral roots. Drawing not to scale.

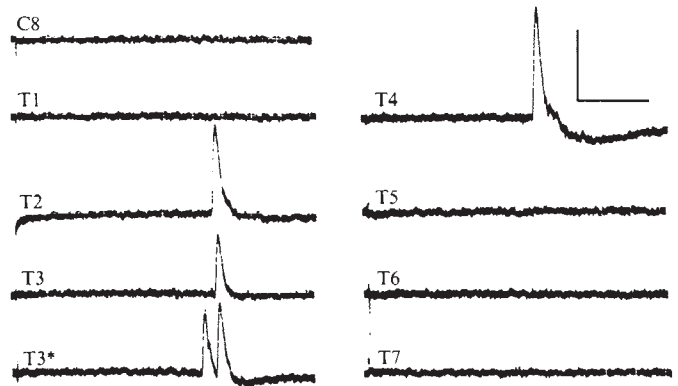


Fig. 2 Synaptic potentials recorded intracellularly from a single fibre in a transplanted intercostal muscle on stimulation of the indicated ventral root. The transplanted muscle, thoracic sympathetic chain, communicating rami and ventral roots were mounted in a bath and superfused with oxygenated Ringer's^{2,3}. The muscle was pinned beyond resting length to minimize its movement. A KCl-filled microelectrode was moved across the implant in small, roughly equal steps, to sample fibres throughout the muscle. For each muscle fibre impaled that had a resting potential of greater than 50 mV (range 50–90 mV) ventral roots C8 to T7 were stimulated in turn with suction electrodes (0.5 ms pulses of ± 5 –50 V at 0.5 Hz). When a response was seen, the strength of the stimulating current was varied to discern multiple inputs from a single ventral root. Reinnervation of the transplanted muscles was often patchy, with nearly all fibres innervated in some areas, and other large areas not innervated at all. However, all muscles tested were reinnervated, and none was excluded on grounds of poor reinnervation. On average, 29 innervated fibres (maximum 35) were studied per muscle. Fibres were generally impaled mid-way along their length, near where the nerve had been implanted, but no other attempt was made to record from near synaptic sites; therefore, synaptic potentials must have been variably attenuated by passive decay. The smallest synaptic potentials we could reliably detect were about 1 mV. Forty-two per cent of unitary inputs were suprathreshold, and gave rise to action potentials. An average of 1.9 inputs (range 1–6) was detected per innervated fibre and 60% of innervated fibres ($n = 941$) received 2 or more inputs. In the fibre illustrated, we recorded four inputs, all of which were subthreshold: one each from T2 and T4 ventral roots, two from T3 (stimulus strength was increased between traces marked T3 and T3*) and none from C8, T1, or T5–7. Calibration bars, 5 mV (vertical) and 50 ms (horizontal).

muscle fibres in the transplant. We thereby compared the segmental origin of inputs to the transplanted T2 and T8 intercostal muscles.

Muscle fibres in both T2 and T8 transplants were reinnervated, often polyneuronally, within 2 weeks (Fig. 2). Figure 3a summarizes the segmental origin of 1,410 inputs to 688 fibres from 11 T2 and 12 T8 muscles studied 2–4 weeks after transplantation. Transplanted T2 and T8 muscles differed in the pattern of the innervation they received: T2 muscles were innervated more effectively by axons from rostral segments, and T8 muscles by axons from caudal segments. Similar results were obtained from muscles examined 10–14 weeks after transplantation (Fig. 3b); thus, neither the existence nor the magnitude of the difference between T2 and T8 muscles is peculiar to an early stage of reinnervation. The ratio of inputs recorded from T2 muscles to inputs recorded from T8 muscle varied progressively from segment to segment (Fig. 3c). Thus, there is a systematic caudal shift in the innervation of T8 compared with T2 muscles.

Because the difference between groups of T2 and T8 transplants was small, it was important to show that the segmental distribution of inputs we observed was a consistent property of individual muscles. We therefore calculated an index of the overall segmental innervation of each transplanted muscle. This index provides a single value equivalent to the average segment of origin of all synaptic inputs recorded; thus, the more caudal the subset of axons that reinnervated a muscle, the higher its

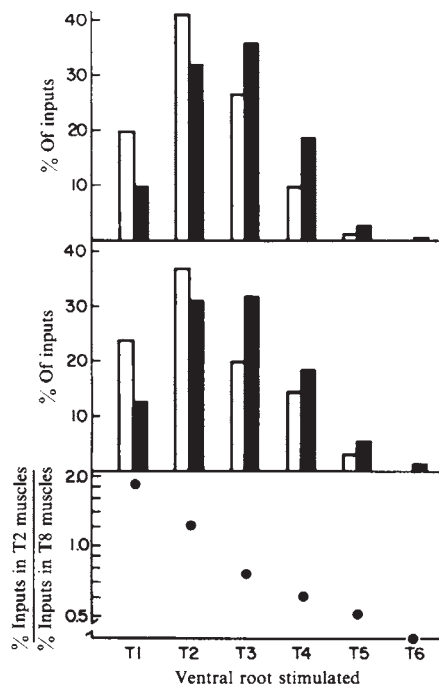


Fig. 3 Reinnervation of transplanted T2 and T8 external intercostal muscles by axons arising from different spinal segments. *a, b*, Per cent of all synaptic inputs recorded from T2 (open bars) or T8 (dark bars) transplants that were evoked by stimulation of the indicated ventral root. *a*, 2–4 weeks after transplantation: 789 inputs recorded from 346 fibres in 11 T2 muscles studied 14–22 days (average 19) after surgery, and 621 inputs from 342 fibres in 12 T8 muscles studied 15–26 days (average 20) after surgery. *b*, 10–14 weeks after transplantation: 235 inputs recorded from 110 fibres in 4 T2 muscles studied 67–97 days (average 79) after surgery, and 331 inputs from 143 fibres in 5 T8 muscles studied 74–96 days (average 80) after surgery. No inputs from C8 or T7 were detected. Segmental distribution of inputs to T2 and T8 muscles differ at $P < 0.001$ in both *a* and *b*, by χ^2 test. *c*, Ratio of inputs recorded from T2 muscle fibres to inputs recorded from T8 muscle fibres, calculated for each segment from the combined data of *a* and *b*.

index of segmental innervation (see Fig. 4 legend). T2 and T8 muscles differed significantly by this index in both the 2–4 ($P < 0.005$) and the 10–14 ($P < 0.05$) week groups (Fig. 4). In 32 experiments, the 10 highest values were all obtained from T8 muscles, and 9 of the 10 lowest values from T2 muscles. Thus, the difference between the innervation of T2 and T8 muscles is reasonably consistent from muscle to muscle.

Many of the synaptic potentials recorded from muscle fibres in the transplants exceeded threshold, initiated propagated action potentials and caused muscle contractions. Forty per cent of inputs to T2 transplants and 44% of inputs to T8 transplants were suprathreshold. An index of segmental innervation calculated using only action potentials showed a difference between T2 and T8 muscles equivalent to that seen when all inputs were considered (Table 1). The difference between T2 and T8 muscles was also demonstrated by visual estimates of the strength of contractions evoked by stimulating individual roots (Table 1). It is possible that the difference between T2 and T8 muscles is reflected in the strength as well as the number of their inputs. However, these results show that the selectivity we observed was neither limited to subthreshold inputs nor influenced by any possible failure to detect small synaptic potentials.

Two further experiments ruled out the possibility that the differential reinnervation of transplanted T2 and T8 muscles was attributable to differences in surgical procedure rather than to intrinsic differences between the muscles. First, in four animals, pieces of both T2 and T8 muscles were removed, but only the T8 muscle was reimplanted. The index of segmental innervation of these transplants (2.77 ± 0.07 ; mean $\pm$ s.e.) was

Table 1 Average segmental innervation of transplanted T2 and T8 intercostal muscles

Measure of reinnervation	Intercostal muscle transplanted	
	T2 (<i>n</i> = 15)	T8 (<i>n</i> = 17)
All inputs	2.32 ± 0.06	$2.76 \pm 0.07^*$
Action potentials	2.24 ± 0.07	$2.79 \pm 0.12^*$
Strength of contractions	2.49 ± 0.08	$2.80 \pm 0.10^\dagger$

Average segmental innervation as judged by all inputs recorded intracellularly was calculated from data presented in Fig. 4. A similar index was calculated for only those inputs that exceeded threshold and gave rise to muscle action potentials. Strength of contractions evoked by single supramaximal stimuli delivered to each ventral root was estimated visually. Responses were rated from 0 (no twitch seen) to 4 (strongest twitch in a given muscle), and a segmental index calculated for each muscle. Differences between values for T2 and T8 muscles are significant at $P < 0.0005$ (*) or $P < 0.02$ (†) by Student's *t*-test.

indistinguishable from that of eight other T8 transplants (2.72 ± 0.14). (This comparison tests for effects of T2 removal; as can be appreciated from Fig. 1, inadvertent damage to relevant structures would have been far more likely to have resulted from removal of T2 than T8 muscles.) Second, the reinnervation of transplanted intercostal muscles was studied in a series of inbred rats (Wistar-Furth) in which one animal served as donor and another as host; again, the muscles of more rostral origin received stronger innervation from more rostral spinal segments (D.J.W. and J.R.S., in preparation).

The selective reinnervation of transplanted intercostals observed here contrasts with the apparently nonselective reinnervation of adult mammalian muscles in several other situations^{7–15}. However, several features of our experiments may

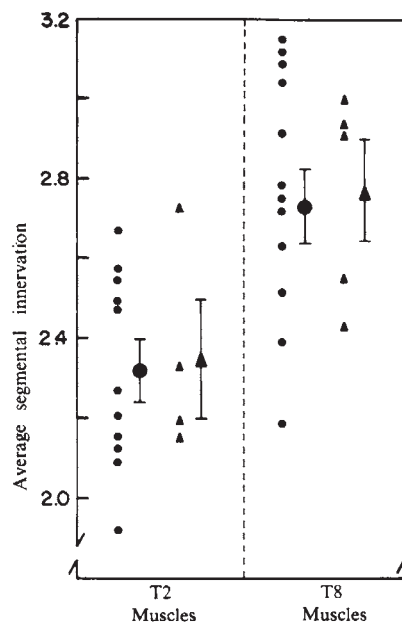


Fig. 4 The average segmental innervation of transplanted T2 and T8 intercostal muscles. This index is the segmentally weighted average of all synaptic inputs recorded from the fibres of a transplanted muscle. For example, a muscle in which we recorded 5 inputs from axons in the T1 ventral root, 20 from T2, 30 from T3, 10 from T4, 5 from T5 and 1 from T6 would be given a value of $(5)1 + (20)2 + (30)3 + (10)4 + (5)5 + (1)6 / 5 + 20 + 30 + 10 + 5 + 1 = 206/71 = 2.90$. Circles represent muscles studied 2–4 weeks after transplantation (see Fig. 3*a*) and triangles represent muscles studied 10–14 weeks after transplantation (see Fig. 3*b*). Small symbols show values for individual muscles, while large symbols show means ($\pm$ s.e.) of groups. The average segmental innervation of the normal superior cervical ganglion, calculated from recordings made by J. Lichtman, is 2.5. Differences between T2 and T8 muscles are significant for both the 2–4 week group ($P < 0.005$) and the 10–14 week group ($P < 0.05$) by Student's *t*-test.

have favoured the detection of selectivity. First, by implanting a single nerve trunk, we ensured that all axons tested had equal and direct access to the target; our results do not depend on the presence or absence of cues on the pathway to the muscle. Second, by transplanting muscles instead of transposing nerves, we were able to compare the reinnervation of nonadjacent muscles. Third, each intercostal is distinct in the segment from which it originates and receives its normal innervation; in contrast, limb muscles used in previous experiments are innervated by^{12,15} and may originate from²³ overlapping sets of segments. Fourth, by comparing inputs from each of several sources (that is, segments) we were able to detect a subtle but systematic difference between the innervation of two muscles. Finally, it is known that preganglionic axons can selectively reinnervate different classes of sympathetic ganglion cells in adults¹⁻⁴; preganglionic axons may reveal differences between adult muscles that motor axons ignore.

We have shown that two adult rat muscles are differentially reinnervated by segmental subsets of a population of autonomic

axons. We conclude that adult muscles can be selectively reinnervated, and predict that T2 and T8 intercostal muscles bear different amounts or types of some factor(s) that contribute to selectivity in synapse formation. In addition, our results raise two possibilities that merit testing. First, the finding that skeletal muscles are selectively innervated by autonomic axons that they normally do not encounter suggests that this selectivity may be based on a recognition system that is widely distributed in the nervous system. Second, the observation that the more rostral of the two muscles we tested is reinnervated by a relatively rostral subset of axons suggests that the basis of the selection could be some positional quality that is shared by nerves and muscles.

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Structural evidence that myosin heads may interact with two sites on F-actin

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Analysis of the rigor complex of muscle thin filaments decorated with myosin subfragment-1 (S-1) by three-dimensional image reconstruction from electron micrographs¹ shows S-1 to be a comma-shaped molecule, the broad head of which interacts with F-actin near the groove between the two strands of actin monomers. Increased resolution near the filament axis has allowed us to distinguish separate components more reliably than in an earlier analysis². Using X-ray diffraction data from striated muscle decorated with S-1 molecules³ as a reference, we have now refined the electron microscope data to show the interaction in more detail. We show here that individual S-1 molecules apparently interact with both strands of a filament, straddling the regulatory protein tropomyosin in the long-pitch groove. This double-sited interaction agrees with chemical cross-linking data⁴ and suggests some interesting possibilities for cross-bridge cycling in muscle.

Optical diffraction patterns from electron microscope (EM) images of different filaments show variation in the positions of intensity peaks along the layer lines, presumably because of distortions induced during staining and dehydration. For example, the radial positions of the strongest peaks on the '1st' and '6th' layer lines ranged from 0.055 nm⁻¹ to 0.080 nm⁻¹ and from 0.035 nm⁻¹ to 0.065 nm⁻¹, respectively, even for our five 'best' images of S-1 decorated thin filaments¹. Originally, a simple average of these data gave peaks centred at 0.0685 nm⁻¹ and 0.0442 nm⁻¹ respectively¹. X-ray diffraction data³ now show the radial positions for hydrated specimens to be 0.060 nm⁻¹ and 0.040 nm⁻¹. Our new average includes only those sets of EM data that closely resemble the X-ray data (Fig. 1). Because of the narrower range of variation, the finer features of the averaged image should be less smeared.

The main peaks of density (Fig. 2, shaded regions) are very similar to those in the original averaged image¹. A division of each actin monomer into two domains (Fig. 3, Aa, Ab), detectable in the earlier image, is now more obvious. The curved end of the S-1 'tail' is somewhat strengthened in density and, finally, a connection between S-1 and an actin monomer on the opposite strand from the main interaction can be seen. The latter was detectable in individual reconstructed images of S-1 decorated thin filaments¹ and also in the averaged image of S-1 decorated pure actin filaments¹. However, being absent from the averaged decorated thin filament image, its significance was previously uncertain. It is not apparent in the reconstructed images of other workers^{5,6} but their specimens appear to have suffered worse distortions than most of ours, as the peaks on the 1st and 6th layer lines are at 0.075-0.100 nm⁻¹ and 0.057-0.080 nm⁻¹ respectively. We attribute the unusual preservation of our best filaments to the method of specimen preparation¹; where distortions are minimized by supporting the filaments on holey carbon, the stain films over holes being stabilized by a final light shadowing of carbon. Minimal dose imaging methods help to avoid stain granulation.

The interaction between S-1 and actin in the rigor state thus seems to involve two independent contacts, in agreement with results obtained by carbodiimide cross-linking of the rigor complex⁴ which indicate that independent regions of the 95,000 molecular weight (MW) heavy chain of S-1 contact two adjacent actin monomers. The most extensive contact in our model (Fig. 3, no. 1) is between S-1 and a monomer on the left of the groove as shown. The weaker contact (Fig. 3, no. 2) is with the monomer just below on the opposite strand. Tropomyosin, although not resolved as a separate entity, appears to interact with S-1 in the fork between its two actin-binding sites. S-1 also has a downward-projecting prong, lying close to the putative tropomyosin position (Fig. 3, S-1c), which may have interfaces with one or both of the next two monomers below. An S-1 molecule bound to four actin monomers could explain a 265,000 MW cross-linked complex sometimes found with the main 170,000 MW complex of one S-1 plus two actin molecules⁴. The latter authors' suggestion that the larger complex consists of two S-1 and two actin monomers seems unsatisfactory as two S-1s cannot bind symmetrically to two actin monomers, because of their staggered polar arrangement (Fig. 3).

The structure analysed represents the rigor state, which has been assumed to correspond to the 'end' of an active stroke. However, X-ray scattering data from S-1 in solution is consistent with this shape, suggesting that the conformation on first binding to actin may also be similar⁷. Nothing is known about conformations at intermediate stages of the cycle; these could be quite different. The apparent existence of two or more independent sites of contact between S-1 and F-actin in the rigor state suggests that the interaction during an active stroke might involve a cycle of different S-1 binding configurations.

Extensive movements are required somewhere in the myosin molecule during muscle contraction. According to the estimates of Huxley and Simmons⁸, each active cross-bridge stroke involves an axial shift of 10 to 12 nm, the distance between one actin monomer and the next but one on the same strand. Probably much of the movement occurs within S-1. The myosin head seems to us a more likely location for primary power generating conformational changes than parts of the molecule

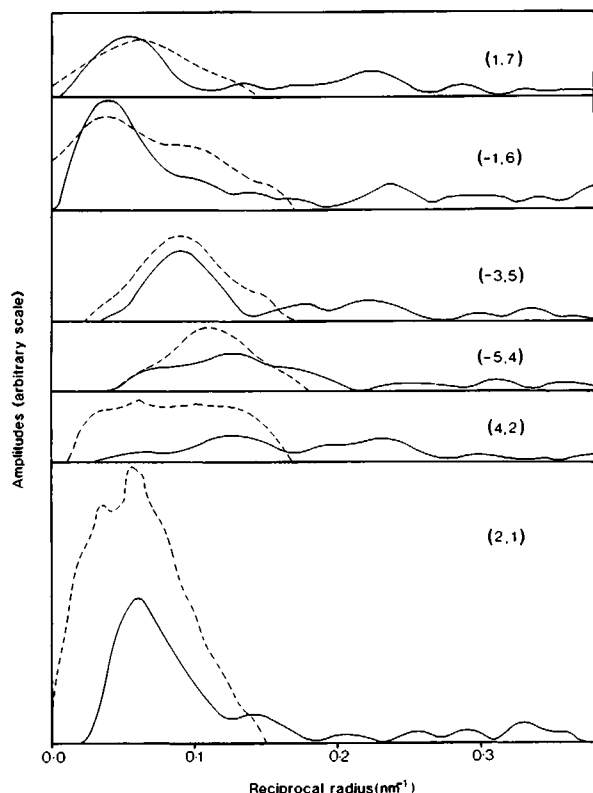


Fig. 1 Layer line amplitudes. The numbers in brackets are n (the Bessel order) and l (the layer line number, based on an axial repeat of $1/39 \text{ nm}^{-1}$ (see ref. 1)). The dashed lines were derived from X-ray diffraction data for insect muscle decorated with papain-cleaved S-1 (ref. 3), the 1st and 2nd layer lines having been folded with a gaussian to remove the Bragg reflections. Data for crab muscle decorated with chymotryptic S-1 are very similar. The solid lines show a new average from EM data derived from nine sides (near and far sides of a helical structure provide nearly independent information in the diffraction pattern¹⁶), including three not used in the original average (ref. 1, Fig. 4). The distribution of peaks remains similar, but there are slight shifts in the positions of the peaks. The corresponding phase changes are also quite subtle. The main differences from the X-ray amplitudes are in the heights and shapes of the peaks on the 1st and 2nd layer lines. If they were weighted up, the main effect would be a strengthening of the tail part of the S-1 molecule. Possibly, the EM reflections are weaker than they should be, owing to disorder at the outermost radii. The additional near-meridional intensities on the X-ray 1st and 2nd layer lines would contribute beyond the radial limits of the EM model. Their presence may reflect some interaction between the S-1 tails and the lattice of thick filaments in the muscle. The equatorial layer line data (not shown) from filaments seen side-on in negative stain are unreliable; nor are these data available from the X-ray patterns because of thick filament interference. A mean radial density distribution for the reconstructed image was therefore calculated by averaging in real space from end-on views of cross-bridges bound to thin filaments. The latter were cut out from computer-filtered images of very thin transverse sections of insect flight muscle (K. A. T., M. K. Reedy and M. Reedy, unpublished data). The result is very similar to the curve deduced from the negatively stained images and used in the original image reconstructions¹.

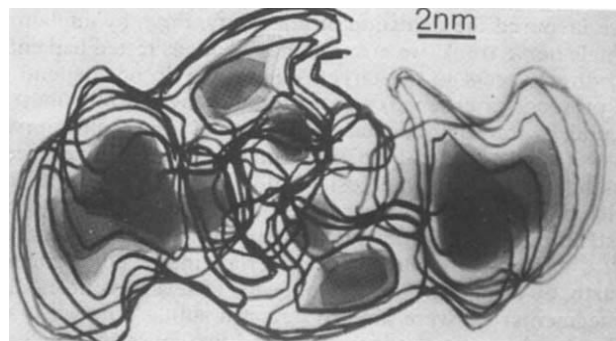


Fig. 2 Part of the three-dimensional reconstructed image, represented by contours drawn on a stack of Perspex sheets. The view is down the filament axis, looking towards the Z-band end. The segment shown includes two actin monomers plus two attached S-1 molecules. The shading indicates the regions of highest stain-excluding density within the reconstructed image. These regions are interpreted in terms of actin, myosin S-1 and tropomyosin as shown in Fig. 3. Actin and S-1 give quite distinct peaks in this view, but tropomyosin is not separately resolved, though its position can be inferred from other evidence¹.

which are distant from the ATP splitting site, or weak (protease sensitive) points such as the S-1/S-2 junction. Evidence from X-ray diffraction is compatible with substantial shape changes in S-1 (ref. 9). A range of different head shapes during contraction could explain the weakening of the actin-based symmetry compared with the rigor state, when a permanent double-sided attachment to actin would stabilize the head in the conformation observed in the EM.

As the regions of strongest density within the reconstructed S-1 structure fall into at least three separate domains (S-1a, S-1b, S-1c), any shape change may primarily involve alterations in angle of 'hinges' covalently connecting different domains, as in tomato bushy stunt virus coat protein where the domains seem relatively rigid¹⁰. There is evidence for one rigid domain at least in the S-1 molecule; Cooke¹¹ has found, using a paramagnetic probe, that the conformation and orientation of an actin-binding domain is unaltered when rigor muscle is stretched. However, other domains may change in internal conformation. So little is known about the structure of S-1 other than in rigor, that ideas on movements during contraction remain purely speculative.

Details of the calcium-regulated control at a molecular level are also unclear. In the active state, tropomyosin occupies a position near the middle of an actin groove^{2,12,13}. In the inhibited state it is probably towards one or other side of the groove^{13,14}, where it could interfere with the interaction between S-1 and actin at either of contact sites 1 or 2. Thus, simple steric blocking is still a possible mechanism. However, Chalovich and Eisenberg¹⁵ have found the association constant of S-1ATP and S-1ADP, P_i with regulated actin to be virtually the same in the

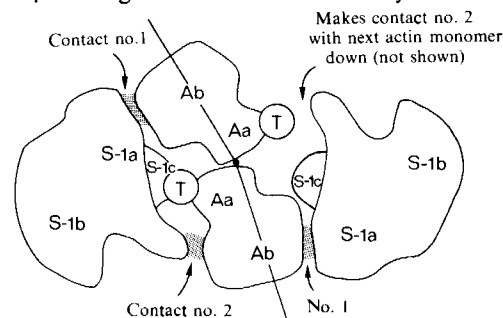


Fig. 3 Diagram of the proposed interactions in the rigor state, between myosin heads (S-1), actin monomers (A) in F-actin and tropomyosin molecules (T) which are thought to lie in the actin helix grooves. Independent sites on S-1 contact two neighbouring actin monomers and there is close contact between S-1 and tropomyosin¹ that may account for the stronger binding of S-1 to regulated than to unregulated actin¹⁷. Each actin monomer consists of two stain-excluding domains, in agreement with other data (for example, ref. 18), S-1 of three such domains. The shape shown here for S-1 gives even better agreement with X-ray scattering data from solutions of the purified protein (R. Mendelson, personal communication) than our earlier model¹.

presence or absence of calcium, although the ATPase rates are very different. They suggest that in the absence of calcium a kinetic step, perhaps release of phosphate, is inhibited. This would also be quite consistent with our model.

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Structure of a Zn^{2+} -containing D-alanyl-D-alanine-cleaving carboxypeptidase at 2.5 Å resolution

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Bacteria possess proteases that are specific for the peptide bonds between D-alanine residues, one of which has a free α -carboxyl group. These D-alanyl-D-alanine peptidases catalyse carboxypeptidation and transpeptidation reactions involved in bacterial cell wall metabolism^{1,2}, and are inactivated by β -lactam antibiotics. We have now elucidated the structure, at 2.5 Å resolution, of the penicillin-resistant Zn^{2+} -containing D-alanyl-D-alanine peptidase of *Streptomyces albus* (Zn^{2+} G peptidase)^{3,4}. The enzyme is shown to consist of two globular domains, connected by a single link. The N-terminal domain has three α -helices, and the C-terminal domain has three α -helices and five β -strands. The Zn^{2+} ion is ligated by three histidine residues, and located in a cleft in the C-terminal domain. The mechanism of action of the enzyme may be related to that of other carboxypeptidases, which also contain functional Zn^{2+} ions.

Crystals of the Zn^{2+} G peptidase were grown⁵, using the vapour diffusion technique, from a solution containing 50 mM Tris-HCl pH 8.0, 5 mM MgCl_2 , 10 mM NaN_3 and a protein concentration of 2%. The crystals were prismatic and belonged to the space group $\text{P}2_1$ ($a = 51.1$ Å, $b = 49.7$ Å, $c = 38.7$ Å and $\beta = 100.6^\circ$). The asymmetric unit contained one protein molecule. The heavy-atom derivatives were prepared by soaking native crystals in appropriate solutions: 5.6 mM $\text{K}_2\text{Pt}(\text{C}_2\text{O}_4)_2$, 3 mM NaAuCl_4 and 30 mM $\text{K}_3\text{UO}_2\text{F}_5$. X-ray diffraction data were collected on a four-circle diffractometer (Hilger-Watts) with a Ni-filtered $\text{CuK}\alpha$ radiation and an ω -scan procedure. Two equivalent reflections for the native and four for the derivatives, including Bijvoet pairs, were measured in shells of increasing θ values. The heavy-atom sites were located using F^2_{HLE} Patterson functions⁶. Heavy-atom parameters were refined by the F_{HLE} procedure, followed by three cycles of phase refinement. The mean figure of merit was 0.66 for 6,700 reflections. Despite this rather low value, the high quality of the electron density map can be judged from Fig. 1.

The interpretation of the 2.5 Å electron density map of the Zn^{2+} G peptidase was carried out more or less simultaneously

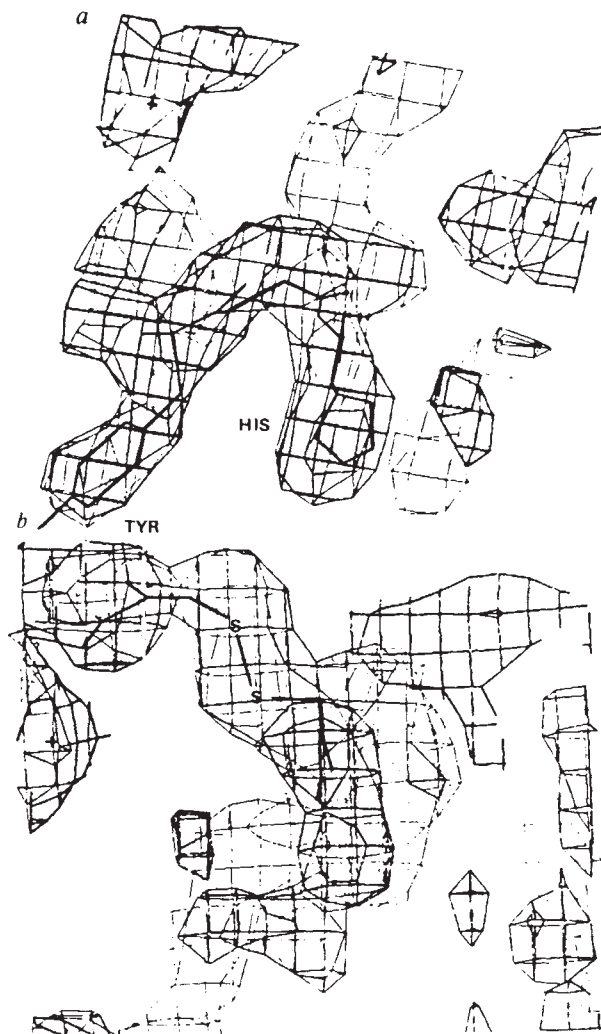


Fig. 1 The electron density map in two different regions of the molecule. *a*, Two large side chains at the end of the cleft: Tyr 154 and His 156. *b*, A well defined S—S bridge: Cys 168—Cys 210.

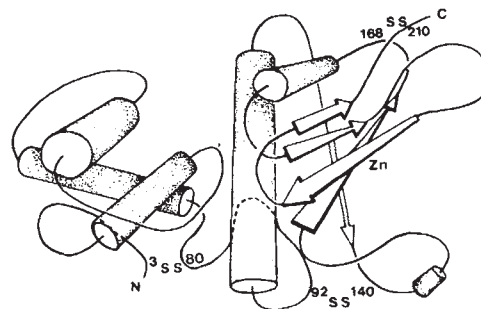


Fig. 2 Schematic drawing of the model of the Zn^{2+} G enzyme built from secondary structure elements. α -Helices and β -strands are represented by cylinders and arrows, respectively. The N and C terminus of the polypeptide chain and the S—S bridges are shown.

with the establishment of its primary structure. Initially, the N-terminal 55 residues and the C-terminal 45 residues could be located directly on the mini-map and six fragments (totalling 84 residues) could be correctly positioned using the Evans-Sutherland graphic system (at the University of London). However, examination of all possible tracings of the polypeptide chain and re-evaluation of the C^α — C^α distances led to the conclusion that a 28-residue fragment was missing. This fragment was subsequently isolated and sequenced so that, at this time, the two techniques are in agreement except for a few residues located outside the catalytic cavity. The complete sequence will be published elsewhere⁷, but Table 1 gives the

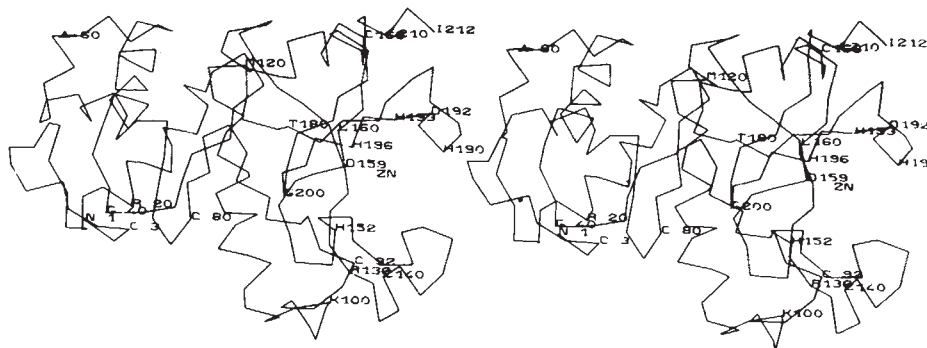


Fig. 3 Stereoscopic views of the main polypeptide chain. The α -carbon positions were derived from the mini-map. Important amino acid residues are labelled using the one-letter code.

positions and proposed functions of some critical amino acid residues.

Besides the Zn^{2+} ion, cystines 92–140 and 168–210 were the most prominent features of the electron density map. Electron density at the methionine residues was rather weak and the weakest Met 153 was located near an Au-binding site. Tracing the polypeptide chain was easy except for two regions, $\text{C}^{\alpha 76}$ – $\text{C}^{\alpha 82}$ and $\text{C}^{\alpha 90}$ – $\text{C}^{\alpha 95}$, respectively. The enzyme molecule has an overall dimension of $48 \text{ \AA} \times 34 \text{ \AA} \times 28 \text{ \AA}$ and consists of two distinct globular domains of different sizes (Figs 2, 3). The small N-terminal domain (76 residues) contains three α -helices (43%) and is connected to the large C-terminal domain (136 residues) by a single link that exhibits a rather unusual folding. The C-terminal domain belongs to the α/β -type secondary structure⁸, containing three α -helices (34%) and five β -strands (17%). About 38% and 11% of the total number of amino acid residues are comprised of α -helix (80 residues) and β -structure (23 residues), respectively. To our knowledge, such a secondary structure has not been found previously in any other protein.

The five β -strands of the C-terminal domain form a mixed sheet with the usual left-handed twist. The cross-over connection is right-handed, a widely observed rule in protein folding⁹. This sheet constitutes the lining of one side of the catalytic cavity. Other remarkable features of the C-terminal domain are the long, 24-residue-containing helix and, especially, the two loops ($\text{C}^{\alpha 186}$ – $\text{C}^{\alpha 193}$ and $\text{C}^{\alpha 138}$ – $\text{C}^{\alpha 152}$) which, in turn, form the edges of the catalytic cavity. This cavity cuts the C-terminal domain into two parts, with the Zn^{2+} cofactor bound inside coordinated with the three protein ligands His 152, His 193 and His 196. Previous studies at $4.5 \text{ \AA}$ resolution¹⁰ have shown this cleft to be the binding site of the two enzyme competitive inhibitors, the dipeptide acetyl-D-Ala-D-Glu and the β -lactam compound *p*-iodo-7- β -phenylacetylaminoccephalosporanic acid. The cavity of the native enzyme was not free of electron density; this might be due either to the presence of ordered solvent molecules or to spurious peaks originating from the heavy-atom binding sites. (In fact, each of the three heavy-atom derivatives studied had at least one binding site inside the cavity.)

A full characterization of the catalytic cavity of the Zn^{2+} G peptidase must await the final fitting of the protein molecule in the Richard's box and crystallographic refinements. Similarly, a full identification of all the residues involved in the binding and catalytic processes must await study at high resolution of various inhibitor (inactivator)–enzyme complexes. Neverthe-

less, at the present stage of the analysis, one may make interesting hypotheses concerning the mechanistic properties of this peptidase. Most likely, Arg 136 is concerned with binding, by charge pairing, of the carboxylate substrate. In addition, two Asp residues (159 and 192) and His 190 are located near the Zn^{2+} cofactor and their side chains have the right orientation to interact with the bound peptide substrate molecule. Contrary to all the other β -lactam compounds tested, β -iodopenicillanate bound irreversibly to the catalytic cavity of the Zn^{2+} G peptidase in rather mild conditions (unpublished results). The difference Fourier synthesis at $2.8 \text{ \AA}$ resolution of the complex thus formed showed two peaks in the vicinity of Arg 136 and His 190, respectively. Permanent inactivation of the peptidase may be due to alkylation of this His residue.

In the proposed catalytic mechanism of carboxypeptidase A and thermolysin^{11–14}, the Zn^{2+} ion functions as a negative-charge stabilizer, the side-chain oxygen of a Glu residue as a proton abstractor (with or without a water molecule as the nucleophile), and a His or Tyr residue as a proton donor. By analogy, one may propose that in the Zn^{2+} G peptidase, Asp 159 or Asp 192 also acts as proton abstractor and His 190 as proton donor. Following this view, the Zn^{2+} G peptidase would be mechanistically similar to the usual metallopeptidases and its specificity would depend entirely on the shape and structural features of its substrate binding cavity.

Two further comments deserve attention. Contrary to observations on carboxypeptidase A, whose active centre is a closed cavity, that of the Zn^{2+} G peptidase is an open cleft. This feature, which is reminiscent of that of thermolysin, should permit accommodation of extended structures. It might be related to the ability of the Zn^{2+} G peptidase to perform endopeptidase activities effectively on complex peptide substrates (possessing a free carbonyl group in α position to the scissile linkage)¹⁵. Finally, the role of the small N-terminal domain remains unknown.

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Table 1 Positions and proposed functions of some critical amino acid residues

Asn–Gly 1–2	N-terminal*
Cystine 3–80	S–S bridge
Cystine 92–140	S–S bridge
Cystine 168–210	S–S bridge
His 152, His 193, His 196	Zn^{2+} ligands
Arg 136	Charge pairing with substrate
Asp 159 or 192	Proton abstractor
His 190	Proton donor
Ile 212	C-terminal

* Partly in the form of a cyclic imide.

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BOOK REVIEWS

Dreams of disappointment

John Maddox

PROFESSOR Victor Jakob, theoretical physicist at a small German university that could be anywhere between Heidelberg and Göttingen, is a creature of the compelling imagination of Professor Russell McCormmach, professor of the history of science at Johns Hopkins University. At the end of his career in 1918, Jakob's spirit has been broken by a succession of disasters — the impending defeat of the Kaiser's reich, his personal defeat and humiliation by the head of the physics institute (an experimentalist), by his sense that the classical values of German society had been corrupted by the synthetic patriotism of the twentieth century and by his failure or, rather, his disinclination to come to grips with quantum physics.

The first appearance on the stage that McCormmach has designed for his protagonist is at a patriotic evening arranged by the university and the local Goethe Society, where he is waiting for the Bach to end to give his first-hand account of heroism in the Franco-Prussian War nearly half a century earlier; before finishing, he collapses with what appears to be a minor stroke. By the end of the book Jakob has sadly wandered off into the woods, fallen into a ditch from which he cannot climb but from which the roar of the guns firing near the university can be heard. Whether the old gentleman succeeds in blowing out his brains with the revolver in his pocket is not known.

In between, McCormmach's Jakob is but a vehicle for a stream of unspoken reminiscence, almost free association, guided chiefly by his wish to understand what has become of himself. The shining hopefulness of his student days had long since drained away. But why had his devotion to classical physics failed to bring the reward of discovery that he and his teachers had every reason, at the outset, to expect? "Why", Jakob keeps worrying, "have I so obviously fallen short of what Hertz, Helmholtz, Drude and those great men expected of me?"

So McCormmach's Jakob is more than a device for telling what physics was like in the early decades of this century. It is also a window on the corrosiveness of disappointment, a way of showing how a person such as Jakob can be destroyed by too great a gap between reality and expectations — of himself and of others. McCormmach says that he has "drawn on an approach from fiction" but he could as well have said that he has written a novel, and a good one at that.

Night Thoughts of a Classical Physicist. By Russell McCormmach. Pp.215. ISBN 0-674-62460-2. (Harvard University Press: 1982.) \$19.50, £10.50.

Jakob's bemusement with his plight is described best by Anna von Helmholtz's account in 1894 of her husband Hermann towards the end of his life:

It is always as if his soul were far, far away, in a beautiful noble sphere where only science and eternal laws rule — and then that does not correspond to anything which surrounds him and he becomes unclear and confused.

Jakob's failure to make a bridge between his internal picture of what the world is like, and the reality outside, is what destroyed him — though reality itself, Jakob as a Jew and as a theoretician, conspired to that end.

Jakob's night thoughts are a moving account of the personal disappointments of academic life in the traditional German academic institute. At the point in his own young career when the next step would have been an appointment as tenured professor (*Ordinarius*), several universities informally enquired of him whether he would be free, but none of them translated this into a formal "call" — the firm offer of a job that, even if declined, could then appear in a *curriculum vitae*. So Jakob soldiered on, becoming indispensable as a teacher (especially when military service dragged away the younger teachers) and was rewarded with the title of honorary professor, an advertisement that he has not quite made the grade.

Before that humiliation, Jakob finds himself being interviewed by the Prussian official Althoff about the prospect of taking over the physical institute in Berlin. Althoff, although only a junior official at the ministry, seems to have established the right to appoint most physics professors at the German universities and to negotiate their terms of service. Among other misdemeanours, he was the principal agent whereby Philip Lenard was foisted on the unwilling faculty at Breslau. Althoff is best known for his complaint that professors are like whores "who go with the one who pays the most". "Each", he is given as saying, "has his price, which is not high".

The account of Jakob's resentment of this interview with the overbearing Althoff is both moving and persuasive, yet it is also one of the points at which McCormmach's technique lets him down. The question why powerful Althoff should be persuading an unsuccessful provincial such as Jakob to

take the post occupied by people such as Helmholtz, Hertz and Drude is a stumbling-block. Worse, at this point the notes tucked away at the back of the book turn out to be as gripping as the narrative.

The most compelling part of Jakob's stream of consciousness is his affection for the great men of German physics — Helmholtz, Hertz, Drude and so on. The tale has it that Jakob's study is decorated with the portraits of his heroes, among whom Maxwell (whom Jakob had never met) and Helmholtz take precedence. But Drude, appointed director of the Berlin institute in 1905, is Jakob's most vivid recollection. A whirlwind of energy, he sought to reorganize the institute but was enmeshed in the tangled bureaucracy of the state — it took endless letters even to change a light-bulb, according to Jakob — so that almost the most tangible of the marks left by Drude on the institute was his habit of keeping his stick of chalk on the left-hand side of the blackboard. Drude himself was driven sick with worry, and to suicide in 1906.

Perhaps predictably, Jakob is better on people than on physics. His stubborn scepticism of the Plancks of his world stems more from aesthetic than from intellectual arguments. He grumbles at the way in which the quantum physicists appear to have been licensed to take any hypothesis as a starting point, making physics seem like mere mathematics. Rather, Jakob argues, just as the study of Latin and Greek is based on the supposed congruence of ancient and modern minds, so classical physics is based on the assumption that at some level there is a congruence between mental and natural processes. But Jakob's refusal to follow Planck, while reinforced by Planck's firm but kind rejection of Jakob's attempt to square the circle of contradiction that had arisen, is at root simply a protest at the way that everything seemed to have changed.

Jakob's stream of reminiscence ends with his fall into the ditch on a hillside high above the university at which his career ended, in the last September of the war. McCormmach, his creator, seems principally concerned to have Jakob describe the social problems of German physics in those stirring days. That his pastiche of a hero should have turned out to be such an appealing down-trodden academic is an uncovenanted benefit, both haunting and memorable. □

John Maddox is Editor of Nature.

Acid tests for the Usanovich theory

John Gibson

A New View of Current Acid-Base Theories. By Harmon L. Finston and Allen C. Rychtman. Pp.216. ISBN 0-471-08472-7. (Wiley: 1982.) £39.50, \$66.50.

MOST chemical reactions and many life processes are carried out in a solvent, and closely connected with the properties of solvents is the behaviour of acids and bases. The acid-base concept has long had a unifying effect in chemistry and in modern times has a great bearing on solvation, compound solubility, the choice of a solvent for chemical synthesis, the understanding of nucleophilic and electrophilic reaction steps, and reaction mechanisms. In their *New View of Current Acid-Base Theories*, Finston and Rychtman review all the old theories, justify them within their limits but finally support the Usanovich theory as the most inclusive, giving experimental evidence for concerted proton and electron transfer, and for the relationship between oxidizing ability and acidity.

The evolution of the acid-base concept over the last three centuries was, at first, a shrinking process, continually reducing the number of substances regarded as acids and bases; it culminated in the restrictive electrolytic dissociation theory of the late nineteenth century, with its emphasis on protons and hydroxide ions in aqueous solution.

Brønsted and Lowry reversed this process in 1923 by omitting water as a requisite solvent and re-defining a base as any substance which can accept a proton. In his book *Valence and the Structure of Atoms and Molecules* published in the same year, G.N. Lewis proposed with complete generality "that a basic substance is one which has a lone pair of electrons which may be used to complete the stable group of another atom . . .". This throwaway line went largely unheeded for 15 years before he supported his alternative ideas in detail. In the meantime the Brønsted-Lowry theory grew strong and an independent theory, the solvent systems theory, became established for aprotic solvents. Only in the late 1930s did Lewis's theory gain currency. Lewis saw no reason for limiting acids to hydrogen-containing compounds and also objected to the over-emphasis of ions in the solvent systems theory. His definition of a base as "have pair, will share" incorporates the Brønsted-Lowry theory but his definition of an acid as an electron pair acceptor continues our enlargement process. The Lewis theory suffers from the disadvantage of being difficult to quantify, but this aspect has attracted attention from Mulliken, Klopman and Hudson, Pearson, Drago, Gutmann and others.

All of this comes over well in Finston and Rychtman's very readable book. Many

comparisons are made between the different theories, but the main message of the book comes in the penultimate chapter. This elaborates the very general definitions of Usanovich who matches the wide definition of base given by Brønsted and Lowry with an equally wide definition of acid, which now becomes capable of splitting off any cation. Moreover, since the electron is regarded as a base, redox reactions become a subclass of acid-base reactions. Thus oxidizing agents relate to acids, reducing agents to bases and quantitative comparisons arise through ionization energies and reduction potentials.

The last chapter is rather novel in that the authors describe some of their own experiments which were designed to test the Usanovich theory as applied to aqueous electrolytes. They carried out pH measurements and acid-base titrations to determine the acidity of the aqueous

oxidization agents dichromate, permanganate and ferric ion. Some support was found by analogous transfer mechanisms for protons and electrons; enhancement of acidity by oxidizing agents; and enhancement of the oxidizing power of a given redox couple with increasing acidity. I think that at this stage the authors would have us believe that our enlargement process is now complete. I take a more conservative view and suggest that some readers might like to repeat these experiments, and perhaps make the obvious extension to non-aqueous solvents, before we can consider the debate finished.

The book is well produced and makes interesting and convincing reading. It would be a good companion to a first-year university lecture course on this subject, but equally would be of interest to researchers in the traditional areas of chemistry and in biochemistry. □

John Gibson is a Senior Lecturer in the Department of Chemistry at Imperial College, University of London.

Unusual chain behaviour

Paul Calvert

Polymers and their Properties. Vol.1, Fundamentals of Structure and Mechanics. By J.W.S. Hearle. Pp.437. US ISBN 0-470-27302-X; UK ISBN 0-85312-033-1. (Halsted/Ellis Horwood: 1982.) \$94.95, £32.50.

IN AN established field the author of a textbook will usually play safe. He will repeat and update the contents of previous books and re-work only those areas which he knows well. Alternative and equally safe techniques are to be abstractly mathematical or to be light and descriptive. Hearle follows none of these courses and has produced a very original and personal book.

He starts with isolated polymer chains and discusses their conformations, energetics and response to stress. The rest of the book then traces the behaviour of the chains in successively more restrained states through melts and solutions, rubbers, glasses and crystals. There is no polymer chemistry, nothing about industrial plastics, no diversions into analytical techniques and not much mathematics, but there is a clear and consistent development of the main theme of chain behaviour. Hearle tries to give simple explanations or analogies for many aspects of chain behaviour and these are accompanied by many rough sketches of chains in various states of distortion.

Some of the questions which he raises have been quietly ignored by most other authors. I think that his explanations are

occasionally wrong but they are usually enlightening. An example is in the discussion of the origin of the β relaxation in polyethylene terephthalate where he draws an analogy between the flexible ethylene moiety and the soft segments of block copolymers. It is a nice idea but β relaxation also occurs in totally rigid molecules and cannot be attributed to a single chain segment.

Also, the final chapter in the book — which tries to relate the morphology of plastics and fibres to their mechanical properties — is not altogether satisfactory. I feel the author fails here because he does not keep a clear distinction between the highly oriented structures of fibres and the isotropic state of plastics where it is no longer fashionable to talk of "fringed micelles". Much of Hearle's own work has been on the structure and properties of fibres, however, so perhaps it is right that the deviations from the mainstream should be greatest here.

I would certainly recommend this book to someone seeking a first course on the physical properties of polymers. It provides a good framework either for more rigorous studies or for understanding the technology and applications of polymers. The small errors can be sorted out later, the overall structure will be of lasting value to a student. □

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Immunological individualism

C.A. Janeway, Jr

Idiotypes and Lymphocytes. By Constantin A. Bona. Pp.211. ISBN 0-12-112950-0. (Academic: 1982.) £18.60, \$28.

IDIOTYPES, which are antigenic determinants unique to antibody molecules binding a particular antigen, have been a central interest of immunologists in recent years. Initial studies defined idiotype determinants immunochemically, and demonstrated that certain idiotype determinants could be used to map genes required for idiotype expression in induced antibody responses. These studies showed that some idiotype determinants behaved as though they were products of germ line genes encoding immunoglobulin heavy and/or light chain variable regions, consistent with the location of these markers.

A second focus has been the use of anti-idiotypic antibodies to probe the nature of lymphocyte receptors for antigen. The precise meaning of such studies is still disputed, but they do support the structural similarity of T and B lymphocyte receptors specific for the same antigen.

Perhaps the most active area of research involving immunoglobulin idiotypes was launched by a theoretical paper published in 1974 by Niels Jerne, in which the immune system was described as a network of interacting idiotypes. The basic premise of the network theory is that immunoglobulins and T cell receptors, since they can specifically recognize virtually any foreign protein, must themselves be as structurally and antigenically diverse as the universe of external or foreign antigens.

It follows from this premise that any foreign antigenic determinant will be mimicked by an internal image antigenic determinant found in the variable domains of an animal's own immunoglobulins and T cell receptors. Furthermore, antibody produced in response to the foreign antigen will also bind to, or be bound by, this internal image set of immunoglobulins and receptors. The circular nature of this interaction has made it difficult to assess its influence on an antibody response, but numerous artificial experiments involving the direct injection of idiotype or anti-idiotypic do suggest that such interactions can affect the expression of idiotype itself.

Because of the extensive interest in idiotype, it would be very helpful to have a current and thorough review of the field, citing major principles and the data that

support them. Unfortunately, Dr Bona has not provided us with such a book. This monograph, though appropriately brief, consists of a good deal of loosely organized data and very little in the way of an enlightening overview. This failing is seen most clearly in the tables and figures, which consist of raw data from Bona's own work. Bona's contributions to this field have indeed been numerous, but valuable data from other laboratories are given short shrift, while those from his own laboratory are presented in detail but without the necessary supporting text, making them virtually impossible to decipher without carefully scrutinizing each table. This greatly weakens the value of the book, as well as using a great deal of space. Furthermore, citations of other authors' research are not very accurate, and, since they are not supported by actual data, the reader seeking information about work by other investigators cannot rely on the text as a source.

This book covers an area of great current interest. But because of its lack of clarity and balance in presentation it can be recommended neither as an introduction to the field nor as a comprehensive treatise intended for the expert. □

C.A. Janeway, Jr is Associate Professor of Pathology at Yale University School of Medicine.

Cell meets cell

David I. Gottlieb

Cell Behaviour: A Tribute to Michael Abercrombie. Edited by Ruth Bellairs, Adam Curtis and Graham Dunn. Pp.615. ISBN 0-521-24107-3. (Cambridge University Press: 1982.) £45, \$89.50.

PROFESSOR Michael Abercrombie was a pioneer in the study of how vertebrate cells move and respond to each other's presence. That line of inquiry has since broadened into a vigorous branch of cell biology which is a major part of contemporary science. As a memorial to the man and his accomplishments, a group of his friends and colleagues have assembled this collection of essays. They have succeeded in both honouring their friend's memory and producing a volume which will be of great use to other scientists.

The book consists of a posthumous review of cell motility by Professor Abercrombie, 22 scientific essays on related topics and a personal memoir of Michael Abercrombie by D. R. Newth.

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Evolution from space

Americans now have the opportunity to read Fred Hoyle and Chandra Wickramasinghe's *Evolution from Space*. The book (which was reviewed in *Nature* 294, 489; 1981) has been published in the United States by Simon & Schuster, price \$13.95.

Most of the essays are critical reviews rather than comprehensive updates of the literature, and many succeed in describing accurately the root observations of the field and critically evaluating our current understanding of the phenomena thus revealed. For this reason the volume should be an enduring one.

Contributions focus on several themes. One is the analysis of how isolated cells move on artificial substrata. Movement of fibroblasts is especially well treated. The topic of polarization of the moving cell as seen by light microscope cinematography is prominent, and the ultrastructural correlates of the light microscopic image are given in detail. A good deal of attention is also devoted to the small-scale specializations of the cell surface which seem to be the actual sites of adhesion, namely focal adhesion plaques and close contact zones. The question of which attributes of artificial substrata are necessary for cell movement and adhesion is reviewed in several of the essays, although the problem of the mechanisms which generate the force necessary for cell motility is not extensively treated. The initial interest in fibroblast movement has since been extended to other types of cells, most notably nerve and epithelia, so it is appropriate that three excellent essays deal with the question of nerve growth cone motility and two with epithelia.

A second theme is the reactions that occur when moving cells in tissue culture encounter one another. Abercrombie's essay reviews the original discovery of contact inhibition between cultured embryonic heart fibroblasts and subsequent efforts to analyse the mechanistic basis for this crucial interaction. The theme of cell-cell interactions is carried over to the interactions of nerve cells with each other and of fibroblasts with epithelial cells, the emphasis being on the morphological complexity of such interactions. The natural stage for cell movement and interactions is the organism; cell and tissue movement in embryonic development is particularly well reviewed. Even better is the account of the migration of cells from the neural crest and of entire tissues during gastrulation, topics which receive a clearer and more comprehensive treatment here than any I have seen in recent years.

This is a valuable book because it gives a clear picture of the current intellectual status of an important part of cell biology and embryology. Part of its message is that there is a wealth of interesting facets of cell behaviour which are accessible to investigation. Armed with all the new probes developed over the past few years (monoclonal antibodies, cloned genes, etc.), molecular biologists will be able to read this book and pick their next set of problems. □

David I. Gottlieb is in the Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis.

Ordered and disordered systems in order

Nicolaas Bloembergen

Nuclear Magnetism: Order and Disorder. By A. Abragam and M. Goldman. Pp.626. ISBN 0-19-851294-5. (Oxford University Press: 1982.) £45, \$69.

PROFESSOR Abragam has scored for the third time in 20 years, thus completing his hat trick. The assist in this case is credited to Dr Goldman. The sport to which this feat applies is the widely diversified field of magnetic resonance; the goals are major publication events scored on the home grounds of the Clarendon Press, Oxford.

Abragam published his first book, *The Principles of Nuclear Magnetism*, already a classic, in 1961. About a decade later, in 1970, he co-authored *Electron Paramagnetic Resonance of Transition Ions* with Professor Bleaney. The present book follows some ten years after the second one. Aficionados of magnetic resonance will not be disappointed. It is a different ball game in the 1980s and there is virtually no duplication with the earlier volumes, amongst which Dr Goldman's *Spin Temperature and Nuclear Magnetic Resonance in Solids* (Clarendon, 1970) should be included. In fact, both this last book and Abragam's *Nuclear Magnetism* are almost required reading before the present volume can be enjoyed. It is definitely not for beginning students.

The first chapter is called "Epitome of the Theory of Spin Temperature" and is a condensation of Goldman's book, with a concomitant high density of equations. It contains the groundwork for all subsequent chapters, which may be read almost independently of one another.

These remaining chapters fall into two parts. In the first the authors discuss three topics, in which they describe themselves as observers rather than active participants. One is concerned with high resolution spectroscopy in solids. The clever manipulation of nuclear spin systems by pulse sequences and by (de)magnetizations in rotating frames of reference leads to uncanny reversibilities, which defy the application of statistical mechanics to nuclear spin hamiltonians in certain cases. The other two topics, concluding the first half of the book, are nuclear magnetism of solid and liquid He₃, respectively. The authors have digested the complex literature about these intriguing quantum solids and liquids, and provide a good introduction to the appreciation of the

fundamental role that nuclear magnetic resonance (NMR) has played in the development of this important sub-field of low temperature physics. The large body of NMR work on solid hydrogen and methane is not discussed, because of the wish of the authors "to complete a book of finite size in a finite time". Although the temperature may be in the order of milliKelvins in some cases, the nuclear spin systems are still largely disordered, as the Boltzmann energy kT remains large compared to the nuclear spin interactions. Nonetheless, in this first part of the book the authors have put a lot of order into the discussion of these disordered systems.

The second part deals with the ordered systems, where the degree of nuclear spin polarization is significantly different from zero, and may approach unity in some cases. The authors have been leaders in a laboratory group that has made most important contributions to this field, so here they perform as actors instead of spectators. They describe the changes in lineshapes, entropy, susceptibilities and other thermodynamic quantities, when the degree of nuclear spin ordering becomes significant. The method of dynamic polarization is discussed in detail: neutrons are scattered from ordered nuclear spin systems, and neutron spin resonance is induced by the pseudomagnetic interaction with the precessing nuclear spin polarization. Again, enough background is provided for the non-specialists in neutron physics.

The final chapter deals with the antiferromagnetic and ferromagnetic ordered arrangements in nuclear spin systems. With the exception of solid He₃ (and nuclear spins in heavy elements, which are not discussed), the ordering is determined by a pure dipolar interaction. This chapter should also be of considerable interest to those who work with the more familiar electronic ferro- and antiferromagnetic materials. The NMR data provide illuminating examples for those more technical fields of magnetism.

In this book a close contact between theory, experiment and data is maintained at all times. The high density of equations is in many spots alleviated by glimpses of physical insight. One would, however, look in vain for detailed descriptions of experimental apparatus.

There is a lot of physics to enjoy in this book. It belongs in all major physics research libraries, as well as in the individual libraries of low temperature physicists and of those active in NMR research or in magnetism. If you enjoyed Abragam and you liked Goldman, you will love Abragam and Goldman. □

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New in paper

Douglas Heggie's *Megalithic Science* (reviewed in *Nature* 296, 373; 1982) has now appeared in paperback. Published by Thames and Hudson, the book costs £5.95. Also new in a paper edition is *Genesis* by John Gribbin. Publisher is Oxford University Press, price £3.95; for review see *Nature* 294, 491; 1981.